

**MEMBRANE EFFECTS OF SEX HORMONES ON GROWTH  
PLATE CHONDROCYTES**

A Dissertation  
Presented to  
The Academic Faculty

By

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In Partial Fulfillment  
of the Requirements for the Degree  
Doctor of Philosophy in Biology

Georgia Institute of Technology  
December 2012

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PLATE CHONDROCYTES**

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## **ACKNOWLEDGEMENTS**

I wish to express my sincere appreciation to my advisor, Dr. Barbara Boyan and Dr. Zvi Schwartz for their supervision, patience, optimism and guidance which has enabled me to successfully complete my thesis. I would like to extend appreciation to my committee members, Dr. Yury Chernoff and Dr. Kirill Lobachev for their support, suggestions, time and encouragement. Special thanks for Dr Roger Wartell, whom this project could never have been completed without. There are some administrators and staff I would like to acknowledge for their help and support through this long journey; Dr. Irma Santoro and Kevin roman for their never ending help in every thing and also being a good source of encouragement.

First of all, I would like to thank Boyan and Schwartz lab, great thanks for all the lab staff that have made the lab function over the years specially: Dr Yun Wang, Srilaxmi Vemula and Lauren Cason. I am also thankful to all the graduate students that have come and gone over the years, but I would like to specifically acknowledge Reyhaan Chaudhri, Rolando Gittens, Jennifer Lynne Hurst-Kennedy and Jung Hwa Park for creating a wonderful laboratory environment, for sharing, caring and being good friends.

My special thanks to my parents without whose love and blessings none of this would be possible. Thank you for your dedication, sacrifice, patience, trust, prayers. I would like to thank my small family, my husband Mohamed who scarified him self for all of us which have brought me thus far. Without him my PhD may never happen. My two young beautiful children Abelrahman and Reim, for their love, encouragement and being such a wonderful children.

Special thanks to Sector Missions of Cultural Relations at the Ministry of Higher Education Arab Republic of Egypt, for supporting me and my family for a complete 4 years. This work was supported by Children's Healthcare of Atlanta, the 401 Price Gilbert, Jr. Foundation.

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## LIST OF SYMBOLS AND ABBREVIATIONS

E <sub>2</sub>	17 $\beta$ -estradiol
ER	Estrogen receptor
DHT	5 $\alpha$ -Dihydrotestosterone
PKC	Protein kinase C
DMEM	Dulbecco's modified Eagle's medium
E <sub>2</sub> -BSA	BSA conjugated 17 $\beta$ -estradiol
PLC	Phospholipase C
RC	Rat costochondral resting zone growth plate chondrocytes
PPT	4, 4', 4''-(4-Propyl-[1 <i>H</i> ]-pyrazole-1, 3, 5-triyl) <i>trisphenol</i>
DPN	Diarylpropionitrile
R, R THC	( <i>R, R</i> )-5, 11-Diethyl-5, 6, 11, 12-tetrahydro-2, 8-chrysenediol
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
$\beta$ -CD	Methyl- $\beta$ -cyclodextrin
PLAP	Phospholipase A <sub>2</sub> activator peptide
GDP $\beta$ S	General G-protein inhibitor
PTX	Pertussis toxin
CTX	Cholera toxin
AR	Androgen receptor
DES	Diethylstilbesterol

## SUMMARY

Understanding and studying the normal bone growth and development is causal. Bone and cartilage tissue provide in addition to their mechanical support, they provide a protection for vital organs such as heart, lung and brain. Longitudinal growth is regulated by the activity of chondrocytes in the epiphyseal growth plates of long bones. Many hormones and growth factors are involved in the regulation of this process. Among these, sex steroids are of crucial importance, especially during puberty.

In long bones, endochondral bone formation occurs at the growth plate, a region of developing cartilage located between the epiphysis and the metaphysis. The process of endochondral ossification is regulated in part by sex steroid hormones. Androgens stimulate endochondral bone growth and elongation, while estrogen is known to suppress longitudinal bone growth and accelerate growth plate closure. Studies using rat costochondral growth plate chondrocytes as a model show that the effects of  $17\beta$ -estradiol ( $E_2$ ) on apoptosis are found in both male and female cells and the same mechanism is involved. In contrast,  $E_2$  causes rapid activation of PKC in female cells but not in male cells. Dihydroxytestosterone (DHT) also has direct effects on growth plate chondrocytes, increasing matrix synthesis including sulfated glycosaminoglycan production, and enhancing cell maturation by increasing alkaline phosphatase enzymatic activity.

Short stature and abnormally slow increase in height is one of the main reasons for referral to endocrinologist. Excessive growth and abnormally tall is also a problem, especially because it increase risk for the trunk abnormalities. Furthermore until now a

few growth-promoting therapies are available for clinical use. Therefore future therapies for treating the growth disorders are essential.

The overall goal of this project is to investigate the sexual-dimorphic effect of the sex steroid hormone in rat growth plate chondrocytes, the cellular signaling pathways mediating these actions, and their physiological role. The information gleaned from this study will provide new information about the role of sex steroid hormones in chondrogenesis and has implications in the development of new therapies for the treatment of bone fracture healing, and growth plate disorders. The central hypothesis was that sex steroid would play an important and sex-specific role in regulating chondrocytes as a main regulator of longitudinal bone growth.

In chapter 2, the hypothesis of sex-specific role of E<sub>2</sub> in chondrocytes was tested by investigating the effect of the active metabolite of estrogen 17 $\beta$ -estradiol (E<sub>2</sub>) on in vitro cultured rat costochondral resting zone chondrocytes (RC). E<sub>2</sub> has previously been shown to regulate rat growth plate chondrocytes through traditional nuclear receptor mechanisms, but only female cells exhibit membrane-associated effects mediated through protein kinase C (PKC)  $\alpha$ . Rat resting zone chondrocytes RCs were isolated from costochondral junction of 150 gram female and male Sprague-Dawley rats. Fourth passage male and female culture were treated with E<sub>2</sub> for 9, 15, 90 minutes and PKC activity was measured. E<sub>2</sub> caused a rapid membrane mediated increase in PKC activity in the female cells within 9 minutes that was maximal at 90 minutes. These results provide definitive evidence that RCs exhibit an intrinsic sex-specific response to E<sub>2</sub> and suggest that sexual dimorphism may be an important variable for cartilage development.

The next aim was to examine the involvement of membrane receptor in  $E_2$  mediated PKC activity in male and female chondrocytes. Fourth passage male and female cultures were treated with the  $E_2$  or its enantiomer Ent- $E_2$  for 9, 15, 90 minutes and PKC activity was measured. Only  $E_2$ , but not *ent*- $E_2$ , caused an increase in PKC specific activity in female RCs, indicating that PKC specific activity is stereospecific and regulated by a specific membrane-mediated mechanism, while supporting the hypothesis that a specific receptor is involved. The involvement of caveolae was also tested by treating the confluent female resting zone chondrocytes with  $E_2 \pm$  methyl- $\beta$ -cyclodextrin ( $\beta$ -CD) (cholesterol removal). The ability of  $\beta$ -CD to abolish the  $E_2$ -mediated PKC activity confirmed the involvement of the membrane-mediated mechanism via caveolae fraction.

The next aim was to determine which ERs were involved in this pathway; we used the same strategy, by treating the female and male cultures with ER $\alpha$ -selective agonist PPT or ER $\beta$ -selective agonist DPN. The cultures were treated for 9, 15, and 90 minutes with PPT or DPN. In addition, female cells were incubated for 90 minutes with  $10^{-7}$  M  $E_2$  in the presence or absence of non-specific antibody to IgG, IgM or with specific antibodies, for ER $\alpha$  and ER $\beta$  respectively. The contribution of ER $\beta$  to any change in PKC activity was determined by treating confluent female cultures with either  $10^{-9}$  to  $10^{-7}$  M selective antagonist/ER $\alpha$ -agonist (R, R-THC) or  $10^{-7}$  M  $E_2$  as positive control for 90 minutes. Alternatively, female RCs were incubated for 90 minutes with  $10^{-7}$  M  $E_2$  in the presence or absence of  $10^{-8}$  or  $10^{-7}$  M (R, R-THC). Both ER $\beta$  and ER $\alpha$  selective agonists DPN and PPT activate PKC at all time points, while the selective ER $\beta$  antagonist/ER $\alpha$ -agonist R, R-THC had no effect suggests that  $E_2$  activates PKC by interacting with both receptor subtypes in female RCs chondrocytes. Our hypothesis was confirmed by the

ability of (R, R-THC) to completely abolish the effect of E<sub>2</sub>-dependent stimulation of PKC. These result indicated that E<sub>2</sub>-mediated PKC activity via both ER $\alpha$  and ER $\beta$ .

In chapter 3, after establishing the involvement of membrane receptor, we then wanted to test the hypothesis if the sex-specific membrane responses of rat costochondral chondrocytes to E<sub>2</sub> are due to the difference in the amount or subcellular location of ER $\alpha$  and ER $\beta$ . Western blots and flow cytometry were used to analyze the expression level and the subcellular location of ER $\alpha$  and ER $\beta$  in both male and female RC cells. Cell fractions of both male and female cells were used to determine the distribution of these two receptors subtypes  $\pm$  treatment of E<sub>2</sub>. Western blots analysis of cell lysates demonstrated the presence of different splicing variants of ER $\alpha$  in both male and female. Western blots of cell lysates demonstrated that both male and female chondrocytes possess ER $\alpha$ . Western blots of the plasma membrane, Caveolae and nuclear fractions of these cells indicated that full length ER $\alpha$  (68KD) as well as truncated isoform ER $\alpha$  (46KD) were present in both cells, While based on the intensity of ER $\alpha$  bands its greatest concentration in female cells. Truncated isoform ER $\alpha$  (36KD) was also present in plasma membrane of female chondrocytes, caveolae and nuclear fraction. However, different from male cells, truncated isoform ER $\alpha$  (36KD) was present only in the nuclear fraction.

The difference of the amount and distribution of ER $\alpha$  confirmed with our flow cytometry result. The number of the plasma membrane ER $\alpha$  in non-permeabilization cells as well as total number of ER $\alpha$  in permeabilization cells indicated that female cells have more ER $\alpha$  compare to the male. Interestingly, flow cytometry data had showed that female chondrocytes had 2 to 3 fold greater number of ER $\alpha$  on the plasma membrane compared to male chondrocytes.

Blots of 50  $\mu$ g protein of male and female cell lysates with ER $\beta$  monoclonal antibody, demonstrated that both male and female chondrocytes express ER $\beta$ . Western blots of the whole cell lysates, plasma membrane, caveolae and nuclear fractions of these cells indicated that full length ER $\beta$  was present in both cells. However, based on the intensity of ER $\beta$  bands, the concentration of the ER $\beta$  in caveolae fraction of female cells is present in higher abundant compare to male cells. Taken together, these result indicated that female chondrocytes expressed more ER $\alpha$  and ER $\beta$  on the membrane than male chondrocytes. The different expression level of ER $\alpha$  and ER $\beta$  between male and female chondrocytes might contribute to their sex-specific membrane responses to E<sub>2</sub>.

To further test the hypothesis that there might be heterodimers complex between both receptors, female cultures were treated for 90 minutes with 10<sup>-7</sup> M PPT in the present or absent of 1:500 dilution of nonspecific antibody IgM or specific antibodies for ER $\beta$ . Alternatively, confluent female cultures were treated for 90 minutes with 10<sup>-7</sup> M DPN in the present or absent of 1:500 dilution of nonspecific antibody IgG or specific antibodies for ER $\alpha$ . ER $\beta$  antibody completely blocked the effect of ER $\alpha$  activator (PPT) to activate PKC. Additionally, ER $\alpha$  antibody completely blocked the effect of ER $\beta$  activator (DPN) to activate PKC. A heterodimeric complex forming between the two receptor subtypes with the treatment of E<sub>2</sub> is confirmed by co-immunoprecipitation result, which indicates the complex formation in female cultures only with the E<sub>2</sub> treatment in contrast to the control. These results support our hypothesis that there might be a heterodimeric complex forming between two receptors with the treatment of E<sub>2</sub>.

To elucidate the pathway, PKC activity was measured in female chondrocytes that were treated with E<sub>2</sub>  $\pm$  U73122 (inhibitor of phospholipase C [PLC]), and PGE<sub>2</sub> level was

measured in female chondrocytes that were treated with  $E_2 \pm$  AACOCF3 (inhibitor of cytosolic phospholipase  $A_2$  [cPLA $_2$ ]). To examine the similarity in the downstream signaling pathway involved in PKC activation between male and female RC cells, male and female cells were treated with *m*-3M3F $\beta$ S (phospholipase C activator) or PLAA peptide (phospholipase  $A_2$  activator) or respectively, and then PKC activity and PGE $_2$  level were measured respectively. Although activation of PKC by  $E_2$  was observed only in female cells, downstream activators *m*-3M3F $\beta$ S induced an increase in PKC activity in both male and female cells, similarly, PLAA downstream PLA $_2$  activators, PLAA peptide induced increase in PGE $_2$  level in both male and female cells. Our study showed that female chondrocytes expressed more ER $\alpha$  and ER $\beta$  on the membrane and caveolae fraction than male chondrocytes while they shared similar signaling pathways.

In chapter 4 and 5 of this study, we hypothesized that the active metabolite of testosterone Dihydrotestosterone (DHT) can induce sex-specific membrane effects similar to other steroid hormones. Confluent cultures of chondrocytes isolated from resting zones of growth plates of both male and female rats were treated with  $10^{-10}$ M to  $10^{-7}$ M testosterone or DHT for 3, 9, 90 and 270 min and protein kinase C (PKC) and phospholipase  $A_2$  (PLA $_2$ ) activities were measured. DHT induced a dose-dependent increase in PKC and PLA $_2$  activity in male cells with the highest increase at  $10^{-7}$  M DHT, whereas testosterone had no effect. PKC activity was augmented at 9 and 90 min, and then decreased to baseline at 270 min. Neither testosterone nor DHT affected PKC in female cells.

To examine the potential signaling pathway involved in PKC activation, male chondrocytes were treated with  $10^{-7}$ M DHT for 9 min in the presence or absence of the



phospholipase C (PLC) inhibitor U73122, the secretory PLA<sub>2</sub> inhibitor quinacrine or the cytosolic PLA<sub>2</sub> inhibitor AACOCF<sub>3</sub>; the G<sub>αi</sub> inhibitor pertussis toxin (PTX) or the G<sub>αs</sub> activator cholera toxin (CTX), and the or the general G-protein inhibitor (GDPβS); an inhibitor of a Ca-ATPase pump in the endoplasmic reticulum (thapsigargin) and inhibitors of specific L type Ca<sup>2+</sup> channels on the cell membrane (verapamil and nifedipine); and an inhibitor of the classical androgen receptor (AR) (cyproterone acetate); as well as the transcription inhibitor actinomycin D, or the translation inhibitor cycloheximide. U73122, quinacrine, and AACOCF<sub>3</sub> inhibited DHT-induced activation of PKC. Inhibition of PLC, G protein, and PLA<sub>2</sub> reduced the response of to DHT in a dose dependent manner, indicating that PLC, G protein and PLA<sub>2</sub> is involved.

After establishing the involved pathway, we tested the effect of DHT on the differentiation and proliferation of male chondrocytes. Treatment of male chondrocytes for 9 minutes with DHT had no effect in [<sup>3</sup>H]-thymidine incorporation in quiescent confluent cultures but caused a dose dependent increase in alkaline phosphatase specific activity.

In summary, our study showed that the active metabolites of sex steroid hormone have a sex specific membrane-mediated affect on chondrocytes. The different expression level of ERα and ERβ between male and female chondrocytes or the ability of complex formation between these two subtypes might contribute to their sex-specific membrane responses to 17β-estradiol. In addition these findings indicate the importance of the membrane effects as mediators of DHT action in chondrocytes and suggest the existence of a specific membrane receptor for DHT in chondrocytes. The information gleaned from this study will provide new information about the role of sex steroid hormones in chondrogenesis and has implications in the development of new therapies for the

treatment of bone fracture healing, and growth plate disorders. The central hypothesis was that sex steroid would play an important and sex-specific role in regulating chondrocytes as a main regulator of longitudinal bone growth.

# **CHAPTER 1**

## **BACKGROUND AND LITERATURE REVIEW**

Cartilage is a connective tissue of predominantly mesodermal origin that provides mechanical support and structural form to many areas of the musculoskeletal system. Cartilage differs from most other tissues due to the fact it does not contain vascular, nervous or lymphatic structures. It is composed of a single cell type referred to as the chondrocyte and a complex extracellular matrix consisting mostly of collagen and proteoglycans.

Estrogen is an important regulator of cartilage biology. Both male and female growth-plate chondrocytes possess ER $\alpha$  and ER $\beta$  [1, 2], E<sub>2</sub>-BSA, which is not hydrolyzed and does not cross the plasma membrane causes many of the same responses as E<sub>2</sub> [3], suggesting that effects of the hormone previously described as traditional nuclear receptor mechanisms may actually result from membrane receptor activation. The effects of E<sub>2</sub>-BSA on PKC activity in rat growth-plate chondrocytes are seen only in female cells [4], suggesting that it is the membrane-associated pathway that determines the sex-specificity of the biological responses to the hormone. This effect is independent of new gene expression and occurs via a pathway that involves G-protein-dependent phosphatidylinositol-specific phospholipase C (PI-PLC) [5]. Similarly, gender-specific activation of PKC has been reported in female human colon cancer cells [6]. PKC appears to be involved in the biological effects of E<sub>2</sub> as well. In growth plate cells, inhibition of PKC with chelerythrine blocks the effects of E<sub>2</sub> on proteoglycan sulfation and differentiation, but has no affect on E<sub>2</sub>-dependent proliferation of these cells. In

contrast, PKC inhibitors block the action of  $E_2$  on DNA synthesis in a number of cell types [7, 8]. Studies using  $E_2$ -bovine serum albumin (BSA) conjugates indicate that it may not be necessary for  $E_2$  to enter the cell to initiate membrane-associated mechanisms [3, 9], suggesting that a membrane receptor may be involved. Moreover the increase in PKC in response to  $E_2$  or  $E_2$ -BSA occurs only in cells from female rats, whereas nuclear receptors are present in male and female cells and neither the ER agonist diethylstilbesterol (DES) nor the ER antagonist ICI 182780 affected  $E_2$ - or  $E_2$ -BSA-stimulated activity [5]. These studies demonstrate the critical role of estrogen receptors in the sex-specific, membrane-mediated effects of  $E_2$  in growth plate chondrocytes.

Two natural potent androgens, testosterone and its metabolite dihydrotestosterone (DHT), are found in humans and mammals [10]. Both androgens interact with the AR to regulate androgen-target gene expression. DHT, which is converted from testosterone by  $5\alpha$ -reductase isozymes, is the major intracellular androgen and the major mediator of androgen actions in the prostate [11]. Like other steroid hormones, testosterone and DHT bind to intracellular ARs, inducing receptor dimerization. This facilitates the ability of ARs to bind to DNA and recruit coregulators to promote or suppress the expression of target genes [12, 13]. This mechanism of steroid action is known as the classical or genomic pathway and because it involves gene transcription and mRNA translation, its effects may take several hours to days to be seen [14].

In addition to their genomic actions, it is now understood that steroid hormones can also exert actions that are insensitive to inhibitors of transcription and translation and occur within seconds to minutes after administration of the hormone [15, 16]. This mechanism of steroid action is referred to as non-genomic or non-classical and may result

in changes in downstream gene expression via membrane-associated signaling pathways [17, 18]. Several studies show that these rapid effects of androgens are mediated by cellular signaling pathways involving surface membrane receptors and second messengers [19, 20]. In cultured cardiac myocytes, testosterone has been found to induce a rapid increase in intracellular  $\text{Ca}^{2+}$  through activation of a plasma membrane AR associated with the pertussis toxin (PTX)-sensitive G protein-coupled phospholipase C (PLC), inositol trisphosphate (IP3) signaling pathway [21, 22]. Similarly DHT induces protein kinase C (PKC) activity via a PLC dependent pathway [23].

Sex steroids can elicit sex-specific effects in their target cells via membrane associated mechanisms. The ability of testosterone or testosterone coupled to bovine serum albumin (T-BSA), which cannot cross the membrane, to increase  $\text{Ca}^{2+}$  in primary osteoblasts is found only in cells from male rats but not from female rats [24]. Importantly, the rapid effect of androgens on PKC in costochondral cartilage cells is specific to DHT and is found only in chondrocytes from male animals. Testosterone does not induce this response in cells from rats of either sex. Estrogens also regulate cells by rapid membrane associated signaling pathways in addition to classical ER-dependent mechanisms [25] and some of the membrane-associated effects are sex-specific [24]. However the exact interaction between androgens and cartilage which plays an important role in skeletal growth remain unclear. It is also uncertain whether these actions have a physiological role. Therefore, the primary aims of this study were to investigate: (A) the rapid actions of DHT in chondrocytes (B) the cellular signaling pathways mediating these actions, and (C) their physiological role in male resting zone chondrocytes.

The model system will be used to investigate the non-genomic actions of sex steroid hormones and their physiological role, primary rat resting zone chondrocytes. In long bones endochondral bone formation occurs at the growth plate, a region of developing cartilage located between the epiphysis and the metaphysis. Endochondral bone formation is regulated in part sex steroid hormone [26, 27]. Androgens stimulate endochondral bone growth and elongation [28] while estrogen is known to suppress longitudinal bone growth and accelerate growth plate closure [29]. The increases observed in periosteal bone formation rates, thus increased bone width, with increased bone elongation would be expected to maintain the proportional shape of the bone. Increased bone elongation and periosteal apposition were observed with DHT treatment even with the administration of estrogen. The effects of androgens on the periosteal bone envelope may have been a consequence of the effects on endochondral growth and ossification. The insufficient levels of certain sex steroids predispose to bone loss and to osteoporotic fractures. For these reasons, resting zone growth plate chondrocytes will be used to study the nongenomic effect of sex steroid hormone and their physiological role.

The mechanism of non genomic effect of sex steroid hormones will be examined in both male and female primary rat resting zone chondrocytes. This study seeks to investigate the underlying cause of the marked differences in physiological responses of male and female rat growth plate chondrocytes to sex steroid hormones, and determine which membrane-associated receptor are responsible for these effects noted in these cells. Collectively, outcomes of this study will provide insights into the cause for rapid membrane sex-specific responses to  $17\beta$ -estradiol in rat growth plate chondrocytes, and non-genomic actions of male steroid hormones in growth plate chondrocytes, the cellular

signaling pathways mediating these actions, and their physiological role. The information gleaned from this study will provide new information about the role of sex steroid hormones in chondrogenesis and has implications in the development of new therapies for the treatment of bone fracture healing, and growth plate disorders.

## CHAPTER 2

### **THE MEMBRANE ASSOCIATED RESPONSE TO 17B-ESTRADIOL IN FEMALE GROWTH PLATE CHONDROCYTES IS MEDIATED BY BOTH $Er\alpha$ AND $Er\beta$ AND REQUIRES PALMITOYLATION TO TRANSLOCATE THE RECEPTORS TO THE PLASMA MEMBRANE**

#### **Introduction**

Estrogens, the main female steroid hormones, are primarily secreted by the ovaries in females and are derived from the testes and extragonadal androgens via aromatization of testosterone and androstenedione in males [30-32]. Estrogens regulate growth, differentiation and function in various target tissues in the human body. Specific receptors for estrogen have been found in growth plate cartilage [33, 34] and fracture callus tissue [35], both of which involve endochondral bone formation.

Estrogens regulate chondrocyte proliferation and differentiation, resulting in growth plate closure during long bone growth [30, 36]. It was thought that these effects were mainly caused by classical nuclear receptors, which act as transcription factors, thereby modulating gene expression [37]. However, recent studies show that membrane-associated signaling pathways are also involved [38-40]

Estrogen receptors  $ER\alpha$  and  $ER\beta$  are members of the nuclear receptor superfamily and their role as ligand-inducible transcription factors has been well established in the literature [41, 42]. In many cells, the receptors coexist either as homodimers or as heterodimers [43], but the subcellular distribution of the two receptors does not quite overlap. A number of variant transcripts have been described for both  $ER\alpha$  and  $ER\beta$ , particularly in cancer cell lines [44]. Several of the variant transcripts of the primary  $ER\alpha$  and  $ER\beta$  pre-mRNA retain the same reading frame as the full-length transcript. While there is considerable information concerning different  $ER\alpha$  splice variants, less is known



about splice variants of ER $\beta$  [45]. Rat ER $\alpha$  splice variants lacking exon 3 and/or 4 (defined as  $\Sigma 3$ ,  $\Sigma 4$  and  $\Sigma 3,4$ ) are all translated into protein in vivo; additionally, their expression levels vary according to the animal's sex [46]. The location of the ER splice variants within the cell and their mechanism of action after hormone binding have been intensively debated. Although full-length ERs are located primarily in the nucleus at steady state, they can shuttle between the nucleus and cytoplasm [47], involving relocation of the hormone-receptor complex [48]. Moreover, the fact that the splice variants lack certain functional domains raises questions about their role in mediating different E<sub>2</sub> effects.

In the classical nuclear receptor mechanism, estrogens cross the plasma membrane and bind to intracellular ERs in the cytoplasm [49]. This induces receptor dimerization, facilitating the translocation of the estrogen/receptor complex to the nucleus where it binds to specific DNA sequences called estrogen response elements, and recruits coregulators to promote or suppress the expression of target genes [50, 51].

Recent evidence suggests that estrogen, as well progesterone and androgens, also induce membrane-associated effects [40, 52-56]. One mechanism occurs when the steroid hormone specifically binds to non-classical transmembrane ERs (i.e., different truncated forms of ERs), which have been found in the plasma membrane of estrogen-sensitive cells [57, 58]. Ligand binding initiates signaling cascades typical of other cell surface receptors, including phospholipid metabolism, Ca<sup>++</sup> ion transport, protein kinase C (PKC) activation and ultimately mitogen activated protein kinase (MAPK) activation [39, 59, 60]. While these signaling pathways can result in altered membrane fluidity by generating lysophospholipids and by retailoring the fatty acid residues [60, 61], steroids

can also modify membrane fluidity via non specific mechanisms [62-64]. Hydrophobic steroids can intercalate into the phospholipid bilayer, with their planar ring system in the hydrophobic core of the plasma membrane bilayer of the target cells [65]. This results in the perturbation of lipid-lipid interactions [65] that may alter the function of membrane proteins and induce an alternate signaling cascade.

Studies using rat costochondral chondrocytes as a model for growth plate cartilage cell maturation [66], have shown that  $E_2$  causes rapid increases in protein kinase C (PKC) activity in female cells via mechanisms that are independent of new gene expression [67]. PKC activation is not affected by the ER antagonist ICI 182780, but can be blocked by antibodies to  $ER\alpha$  [5], suggesting that non-traditional mechanisms may be involved. Moreover,  $E_2$  elicits a rapid increase in PKC when incubated directly with isolated plasma membranes and cells treated with bovine serum albumin conjugated  $E_2$ , which cannot enter the cell, also exhibit rapid increases in PKC [68, 69]. This indicates that specific receptor(s) are involved and that they are present in the plasma membrane.

Although different structural and functional properties have been reported for the plasma membrane-associated ERs by comparison with the nuclear  $ER\alpha$  and  $ER\beta$  [70, 71], immunocytochemical studies have revealed the presence of a significant fraction of nuclear ER also in the plasma membrane [72, 73]. The growth plate chondrocytes possess both  $ER\alpha$  and  $ER\beta$  [74, 75] and recent studies show that  $ER\alpha$  is present in the plasma membrane in three molecular forms: the traditional 66kDa nuclear form ( $ER\alpha_{66}$ ), a 46kDa form ( $ER\alpha_{46}$ ) and a 36kDa form ( $ER\alpha_{36}$ ) [45]. It is not well understood how ERs are maintained or translocated to the plasma membrane. ERs do not possess an intrinsic transmembrane domain [76], suggesting that they interact with specific

membrane protein(s) [77, 78]. Caveolin-1 has been implicated as has palmitoylation as a mechanism for locating ERs at the plasma membrane [79-81].

The goal of this study was to determine the specific roles of ER $\alpha$  and ER $\beta$  in the rapid membrane-associated E<sub>2</sub>-dependent increase in PKC. To do this, we used the growth plate chondrocyte model described above. The direct receptor binding and indirect membrane perturbation effects of the steroid on receptor function can potentially be distinguished by observing the differences in the effect of each E<sub>2</sub> enantiomer on cell response [82]. Accordingly, we determined whether the membrane response was mediated by specific receptor/ligand interactions by comparing the effect of E<sub>2</sub> and its enantiomer *ent*-E<sub>2</sub>. We used selective agonists and antagonists of ER $\alpha$  and ER $\beta$ , as well as specific blocking antibodies to determine if one or both receptors mediate the response. Finally, we determined if palmitoylation or myristylation of the receptor(s) is involved in targeting the receptor(s) to the plasma membrane.

## **Materials and Methods**

### ***Reagents***

17 $\beta$ -estradiol was purchased from Sigma Chemical Co. (St. Louis, MO). *ent*-17 $\beta$ -estradiol was prepared as described earlier [83]. ER $\alpha$  monoclonal antibody (ab16460), ER $\beta$  monoclonal antibody (ab16813), non-specific IgG (ab91353), and non-specific IgM (ab91545) were purchased from Abcam (San Francisco, CA). ER $\alpha$ -selective agonist propyl pyrazole triol (PPT), ER $\beta$ -selective agonist diarylpropionitrile (DPN), and ER $\beta$ -selective antagonist/ER $\alpha$ -agonist (R,R-tetrahydrochrysene [R,R-THC]) [84-86] were obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Tunicamycin, an inhibitor of palmitoylacyltransferase, [38] was purchased from EMD Chemicals, Inc. (San Diego,

CA, USA). 2-Hydroxymyristic acid (HMA), a myristate antagonist [38], was obtained from Cayman Chemical (Ann Arbor, MI). A PKC BioTrak assay kit was obtained from GE Lifesciences (Piscataway, NJ) and Dulbecco's modified Eagle medium (DMEM) was obtained from GIBCO-BRL (Gaithersburg, MD). A PLA<sub>2</sub> assay kit was obtained from Cayman Chemical (Ann Arbor, MI). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay kit [87] obtained from Pierce Chemical Co. (Rockford, IL). [<sup>32</sup>P]-ATP was obtained from Perkin Elmer (Melville, NY).

### ***Chondrocyte Cultures***

The culture system used in this study has been described in detail previously [88]. Chondrocytes were isolated from the resting zone of the costochondral junction of 150 gram female Sprague-Dawley rats. To eliminate the contamination by fibroblasts and osteoblasts, perichondrial tissue and calcified cartilage were discarded. After dissection, the cartilage was thinly sliced and cells were released from the tissue by sequential incubations in 0.25% trypsin-EDTA for 1 hour after which the initial digest was discarded to further eliminate contaminating cells. Pure chondrocyte isolates were then obtained by digestion in 0.02% Type II collagenase for 3 hours in Hank's balanced salt solution. Chondrocytes were plated at an initial density of 10,000 cells/cm<sup>2</sup> and maintained in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml ascorbic acid in an incubator at 5% CO<sub>2</sub> and 100% humidity at 37°C. The culture medium was replaced after 24 hours and then every 48 hours until cultures reached confluence. Fourth passage cells were used for all experiments based on previous studies showing that these cells preserve their chondrogenic phenotype as well as their differential responsiveness to vitamin D metabolites at this passage [89].

### ***ER $\alpha$ /ER $\beta$ Complexes***

In order to determine if ER $\alpha$  and ER $\beta$  form a complex, confluent chondrocyte cultures were incubated for 90 minutes with  $10^{-7}$  M E<sub>2</sub>, and a monoclonal antibody to ER $\alpha$  were used to immunoprecipitate the receptor and associated proteins from cell lysates. At harvest the cell layers were washed with PBS and the cells were lysed and sonicated in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM disodium EDTA, 1% Nonidet P-40) containing 100 mM NaF, protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein samples were mixed with either anti-ER $\alpha$  antibody and incubated at 4°C overnight with continuous agitation. Immunoprecipitation was measured in protein samples using a commercially available Dynabeads® Protein kit following the manufacturer's directions (Invitrogen, Grand Island, NY). The immunoprecipitated samples were then subjected to Western blot analysis. Whole cell lysates and the immunoprecipitated proteins were separated on a 4-20% gradient acrylamide gel. Western blots of the gels were probed with either the mouse monoclonal antibody to ER $\alpha$  (ab16460) or anti-ER $\beta$  monoclonal antibody (ab16813). In order to lower the background in the images, a ONE-HOUR IP-Western Kit (Genscript) was used. Immunoreactive bands were visualized using goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). The Super Signal West Pico Chemiluminescent System (Thermo Fisher Scientific) was used to treat the membrane and then imaged with the VersaDoc imaging system (Bio-Rad).

### ***Regulation of Protein Kinase C Activity***

Initial experiments determined the time course of E<sub>2</sub> and *ent*-E<sub>2</sub> regulation of PKC activity. Confluent fourth passage female cells in 24 well plates were treated for 9, 15 and

90 min with 0.5 ml of ethanol at highest concentration used in experimental cultures or experimental media (DMEM + 10% FBS + various concentrations  $E_2$  or *ent*- $E_2$ ). Dose response was determined by treating female cells for 90 minutes with  $10^{-9}$  to  $10^{-7}$  M  $E_2$  or *ent*- $E_2$ . In order to determine which ERs were involved in this pathway, we used the same strategy, by treating the female cultures with ER $\alpha$ -selective agonist PPT or ER $\beta$ -selective agonist DPN. The cultures were treated for 9, 15, and 90 minutes with PPT or DPN. Alternatively, dose response was determined by treating female cells for 90 min with  $10^{-9}$  to  $10^{-7}$  M PPT or DPN.

The contribution of ER $\beta$  to any change in PKC activity was determined by treating confluent female cultures with either  $10^{-9}$  to  $10^{-7}$  M selective antagonist/ER $\alpha$ -agonist (R,R-THC) or  $10^{-7}$  M  $E_2$  as positive control for 90 minutes. Alternatively, female RCs were incubated for 90 minutes with  $10^{-7}$  M  $E_2$  in the presence or absence of  $10^{-8}$  or  $10^{-7}$  M (R,R-THC).

In addition, female cells were incubated for 90 minutes with  $10^{-7}$  M  $E_2$  in the presence or absence of non-specific antibodies IgG and IgM or with specific monoclonal antibodies to ER $\alpha$  and ER $\beta$ , respectively. Antibodies were added to the cultures at a 1:500 dilution. Although these antibodies are specific for the intracellular ERs, the anti-ER $\alpha$  antibody has been used successfully to label protein in the plasma membrane [90].

To further evaluate if both ER $\alpha$  and ER $\beta$  are involved, and to test the hypothesis that there might be a heterodimers complex between them, female cultures were treated for 90 minutes with  $10^{-7}$  M PPT in the presence or absence of a 1:500 dilution of IgM or of the anti-ER $\beta$  antibody. Alternatively, confluent female cultures were treated for 90

minutes with  $10^{-7}$  M DPN in the presence or absence of 1:500 dilution of IgG or the anti-ER $\alpha$  antibody.

To assess the role of palmitoylation or myristylation of ERs in regulating PKC activity, female cells were pre-incubated for 2 hours with 30 $\mu$ M tunicamycin [57] or with 0.5mM 2-hydroxymyristic acid (HMA) [57], and then with  $10^{-7}$  M E<sub>2</sub> for 90 minutes. In addition, to determine the specific role of palmitoylation of either ER $\alpha$  or ER $\beta$ , female chondrocyte cultures were treated for 2 hours with 30 $\mu$ M tunicamycin and then with either  $10^{-7}$  M PPT or  $10^{-7}$  M DPN for 90 minutes.

After the incubation period, cell layers were washed with PBS to remove any excess medium and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1% NP-40). 25 $\mu$ l of the cell lysates were assayed for protein content [87]. PKC activity was measured in cell lysates using a kit following the manufacturer's directions (Amersham Bioscience). This kit is optimized for measurement of phospholipid and Ca<sup>2+</sup>-dependent PKC [91, 92].

### ***Regulation of Phospholipase A<sub>2</sub> Activity***

To determine if either or both receptors mediate the effects of E<sub>2</sub> on PLA<sub>2</sub> activity, the following protocol was used. For each experiment, confluent cultures of female chondrocytes in 24 well plates were treated for 90 minutes with 0.5 ml of the control medium (0.02% ethanol in DMEM + 10% FBS) or experimental medium (DMEM + 10% FBS + various concentrations of E<sub>2</sub>). After the treatment period, cell layers were washed with PBS and then cells were sonicated in 0.1 ml of cold buffer (50 mM 4-2 hydroxyethyl-1-piperazineethane sulfonic acid containing 1 mM EDTA). Cell pellets were obtained by centrifugation for 15 minutes. 25 $\mu$ l of the cell lysates were

assayed for protein content. PLA<sub>2</sub> activity was measured in cell supernatant using a kit following the manufacturer's directions (Cayman Chemical). Samples were analyzed using a plate reader at 414 nm [61, 93, 94].

To better understand the role of ERs in regulating PLA<sub>2</sub> activity, confluent female cultures were treated for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> in the presence or absence of the anti-ER $\alpha$  antibody or nonspecific mouse IgG. Alternatively, female cultures were treated for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> in the presence or absence of anti-ER $\beta$  antibody.

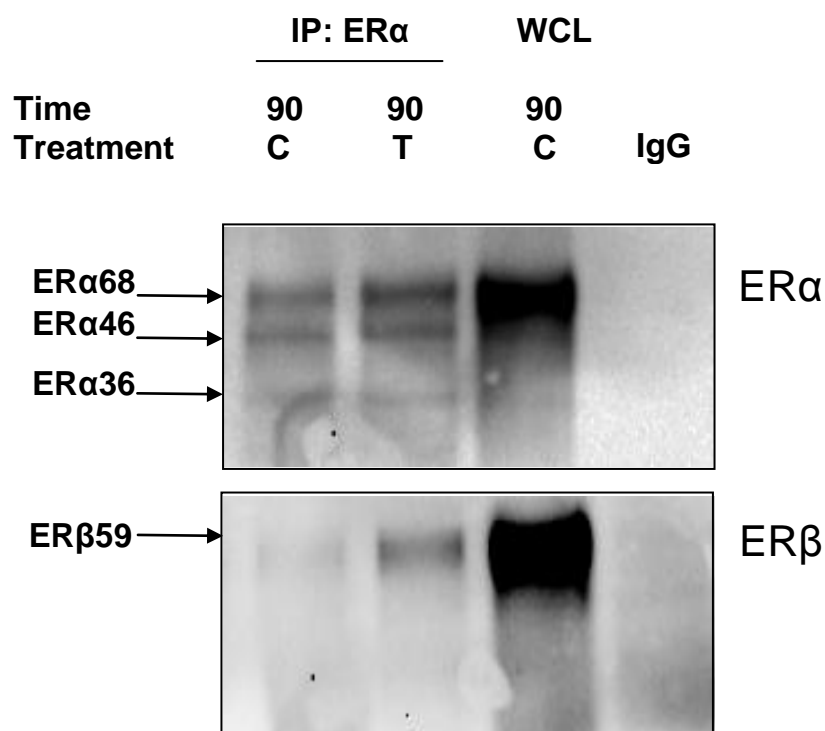
### ***Statistical Management of the Data***

For each experiment, each value represents the mean  $\pm$  SEM of the cell layers of six independent cultures. Statistical significance was determined by ANOVA; P<0.05 was considered significant. Each experiment was repeated two or more times to ensure the validity of the results. The data presented are from a single representative experiment.

### **Results**

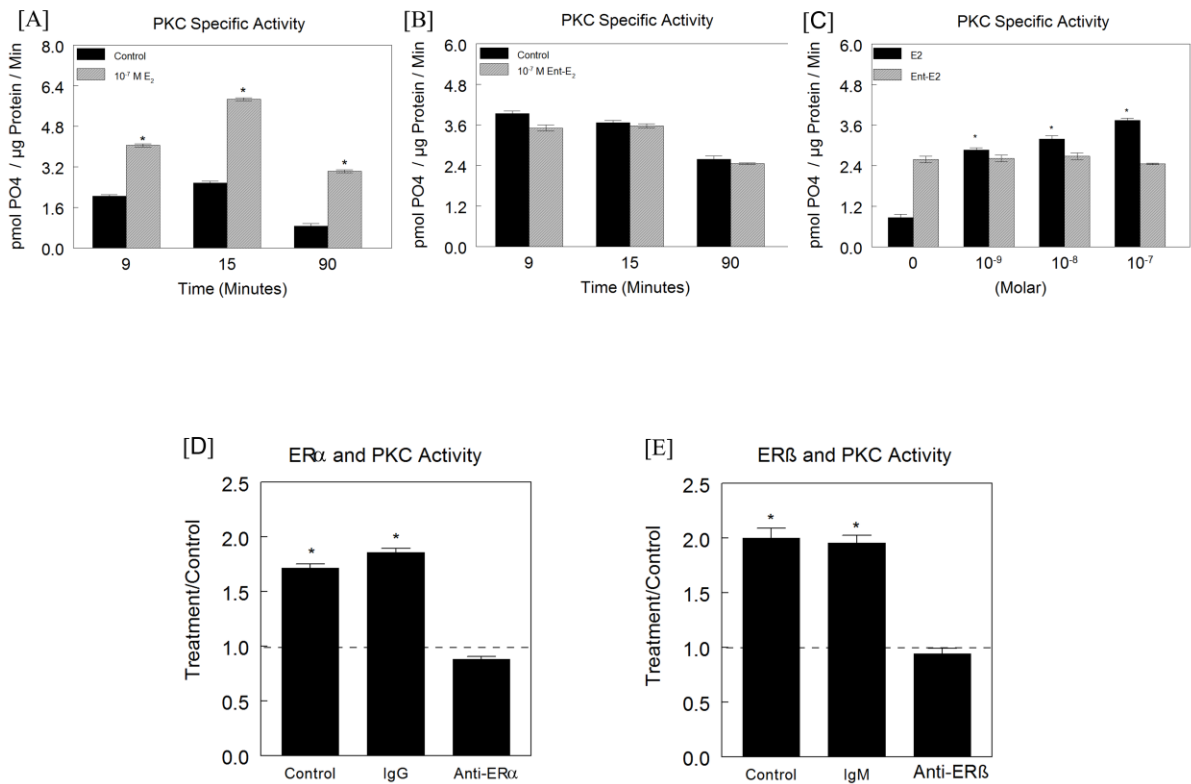
Female resting zone chondrocytes possess ER $\alpha$  and ER $\beta$  (Figure. 2.1). Western blots of whole cell lysates demonstrate the presence of ER $\alpha$ 68, ER $\alpha$ 46, and ER $\alpha$ 36 as well as ER $\beta$ 59. Immunoprecipitates of the lysates using antibodies to ER $\alpha$  confirm that ER $\alpha$ 68, ER $\alpha$ 46, and ER $\alpha$ 36 are present in control cultures, but the amount of ER $\beta$ 59 is considerably reduced in comparison to the whole cell lysates. Treatment of the cells with E<sub>2</sub> for 90 minutes increased the amount of ER $\alpha$ 68, ER $\alpha$ 46 and ER $\beta$ 59 in the anti-ER $\alpha$  antibody immunoprecipitates, suggesting that they have formed a complex.





**Figure 2.1: ER $\alpha$  was immunoprecipitated and subjected to Western blot.** The membranes were incubated with the anti-ER $\alpha$  and anti-ER $\beta$  antibodies. C: control group, T: E<sub>2</sub> treated group.

The effect of E<sub>2</sub> on PKC is receptor-mediated. Only E<sub>2</sub>, but not *ent*-E<sub>2</sub>, caused an increase in PKC specific activity in female RCs (Figure 2.2A, B). A 2-fold increase in PKC activity was evident at 9 minutes, a 2.4-fold increase at 15 minutes and a 3.5-fold increase at 90 minutes in response to E<sub>2</sub>. The effects of E<sub>2</sub> were dose-dependent with the greatest increase in PKC activity observed in cells treated with 10<sup>-7</sup>M E<sub>2</sub> (Figure 2.2C). Antibodies to ER $\beta$  or ER $\alpha$  blocked the E<sub>2</sub>-stimulated PKC activity completely whereas non-specific IgM and IgG had no effect (Figure 2.2D, E), suggesting that both receptors were involved.



**Figure 2.2: Time course and dose-dependent effect of E<sub>2</sub>, *ent*-E<sub>2</sub> and role of ERs in PKC specific activity in female resting zone chondrocytes.** Time course was determined by treating chondrocytes for 9, 15, and 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> (A) or 10<sup>-7</sup> M *ent*-E<sub>2</sub> (B). Dose dependent effects were determined by treating female cells for 90 minutes with 10<sup>-9</sup> to 10<sup>-7</sup> M E<sub>2</sub> or *ent*-E<sub>2</sub> (C). Role of ERs was tested by treating the cells for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> ± anti-ERα antibody (D) or E<sub>2</sub> ± anti-ERβ antibody (E). PKC specific activity was measured in cell layer lysates. Values are the mean ± SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control at each time point.

Both the ERα-selective agonist PPT and the ERβ-selective agonist DPN activated PKC at all time points examined (Figure 2.3A,B) and both agonists exhibited comparable dose-response effects at 90 minutes (Figure 2.3C). PKC activity was not significantly altered in chondrocytes treated with 10<sup>-9</sup>-10<sup>-7</sup> M (R,R-THC), which is an agonist for ERα and an antagonist for ERβ (Figure 2.3D). However, R,R-THC blocked the stimulatory effect of E<sub>2</sub> (Figure 2.3E).

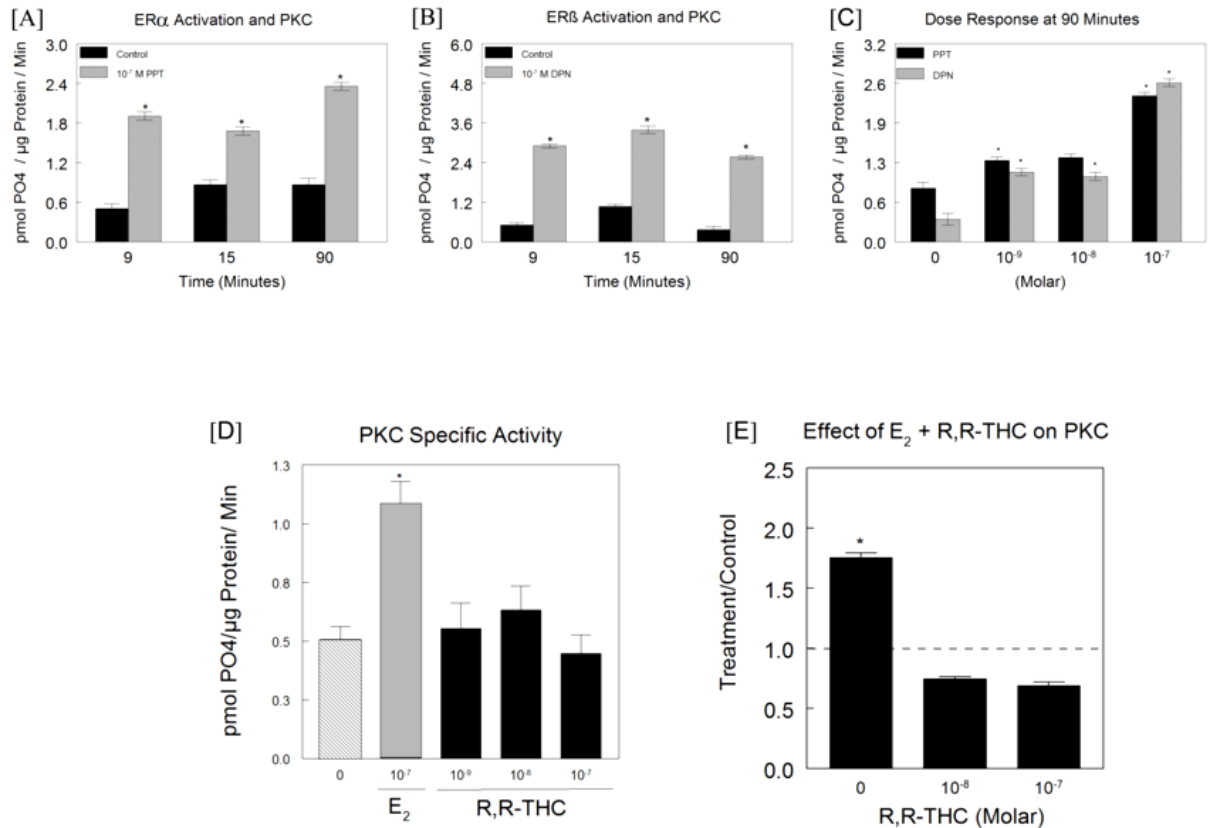
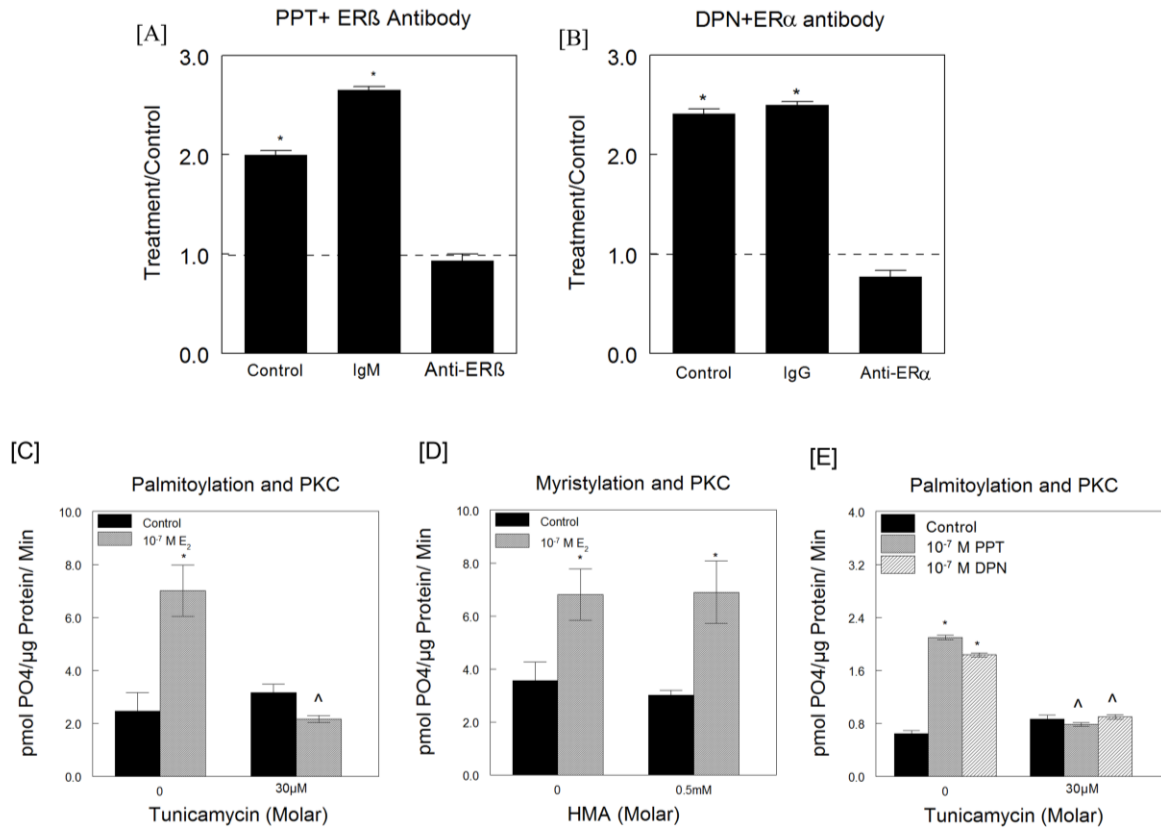


Figure 2.3: Time course and dose-dependent effect of ER $\alpha$  and ER $\beta$  agonists on PKC specific activity in female resting zone chondrocytes. The time course was determined by treating the chondrocytes for 9, 15, and 90 minutes with 10<sup>-7</sup> M PPT (A) or 10<sup>-7</sup> M DPN (B). Dose dependent effects were determined by treating female cultures for 90 minutes with 10<sup>-9</sup> to 10<sup>-7</sup> M PPT, DPN (C). The role of ER $\beta$  was determined by treating the chondrocytes with 10<sup>-9</sup> to 10<sup>-7</sup> M R,R-THC or 10<sup>-7</sup> M E<sub>2</sub> as a positive control (D) or with 10<sup>-7</sup> M E<sub>2</sub>  $\pm$  10<sup>-8</sup> to 10<sup>-7</sup> M R,R-THC (E). PKC activity was measured in cell layer lysates. Values are the mean  $\pm$  SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control

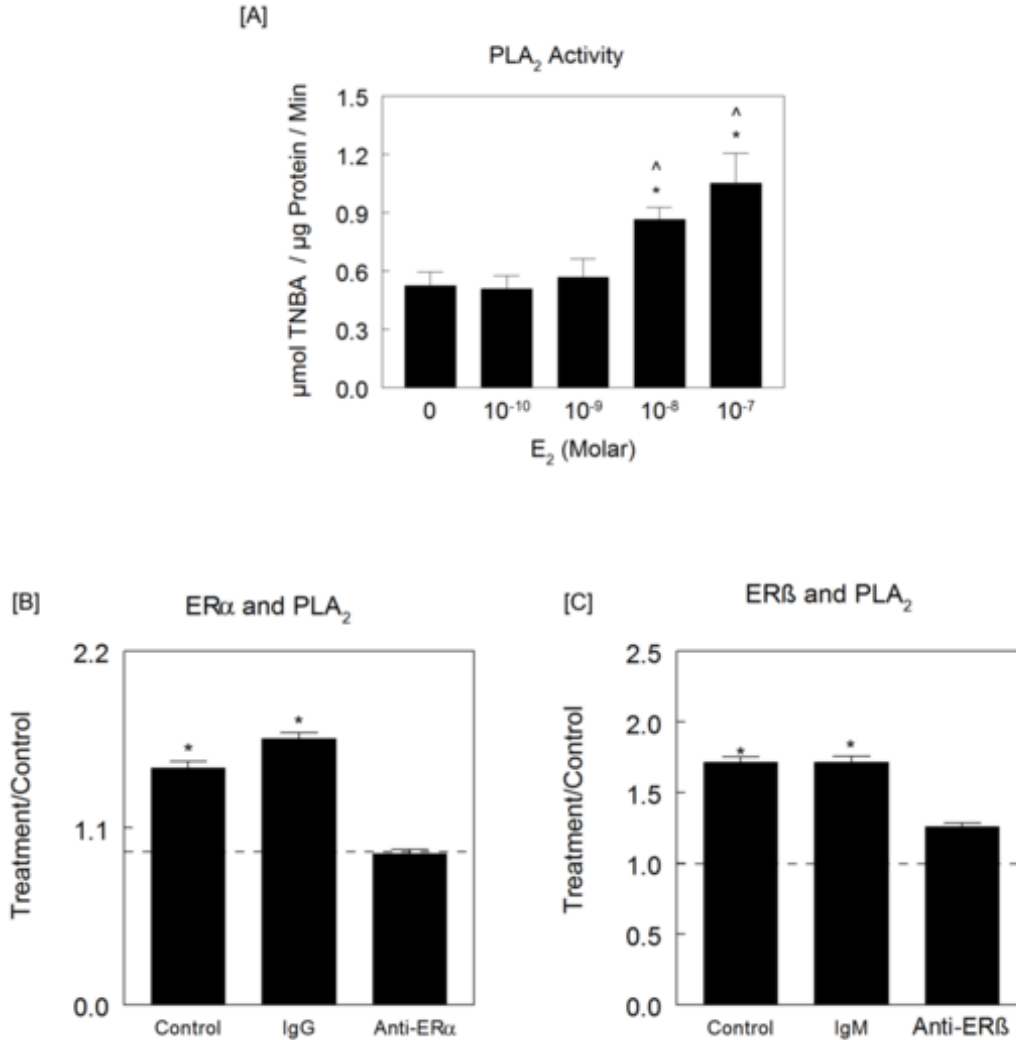
Treatment of the chondrocytes with antibodies to ER $\beta$  completely blocked the stimulatory effect of the ER $\alpha$  agonist PPT on PKC; and antibodies to ER $\alpha$  blocked the effect of the ER $\beta$  agonist DPN (Figure 2.4A,B). Tunicamycin (30 $\mu$ M) was able to completely abolish the effect of E<sub>2</sub> on PKC activity whereas HMA had no effect (Figure 2.4C,D), indicating that palmitoylation but not myristylation was required. Additionally,

tunicamycin blocked the stimulatory effect of PPT and DPN on PKC specific activity (Figure 2.4E).



**Figure 2.4: Involvement and palmitoylation of ER $\alpha$  and ER $\beta$  are required in E<sub>2</sub>-mediated PKC activity.** Female cultures were treated for 90 min with 10<sup>-7</sup>M PPT  $\pm$  anti-ER $\beta$  antibody (A), or for 90 minutes with 10<sup>-7</sup>M DPN  $\pm$  anti-ER $\alpha$  antibody (B). Effect of palmitoylation of ERs on E<sub>2</sub>-dependent PKC activity: Female cultures were pre-incubated for 2 hours with 30 $\mu$ M tunicamycin (C), or 0.5mM 2-hydroxymyristic acid (D). Then cells were treated for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub>. The same strategy was used by pre-incubating the cells for with 30 $\mu$ M tunicamycin (E); then cells treated for 90 minutes with 10<sup>-7</sup>M PPT or DPN. PKC specific activity was measured in cell layer lysates. Values are the mean $\pm$ SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control, <sup>^</sup>P<0.05, E<sub>2</sub>+tunicamycin vs. E<sub>2</sub> alone or PPT + tunicamycin vs. PPT or DPN + tunicamycin vs. DPN alone for each experiment.

E<sub>2</sub> dependent signaling via PLA<sub>2</sub> was also regulated by the two receptors. Chondrocytes treated with E<sub>2</sub> exhibited increased PLA<sub>2</sub> specific activity (Figure 2.5A) and a 1: 500 dilution of antibodies to either ERβ or ERα completely blocked this effect whereas IgM and IgG had no effect (Figure 2.5B,C).



**Figure 2.5: Involvement of ERα and ERβ in E<sub>2</sub>-mediated PLA<sub>2</sub> activity.** Confluent female resting zone chondrocytes were treated for 90 minutes with 10<sup>-10</sup> to 10<sup>-7</sup> M E<sub>2</sub> (A), or with 10<sup>-7</sup> M E<sub>2</sub> ± anti-ERα antibody (B), or with 10<sup>-7</sup> M E<sub>2</sub> ± anti-ERβ antibody (C). PLA<sub>2</sub> specific activity was measured in cell layer lysates. Values are the mean ± SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control, ^P<0.05, 10<sup>-10</sup> M vs. 10<sup>-8</sup>-10<sup>-7</sup> M E<sub>2</sub>. Involvement of ERα and ERβ in E<sub>2</sub>-mediated PKC activity: Female cultures were treated for 90 min with 10<sup>-7</sup> M PPT ± anti-ERβ antibody (D), or for 90

minutes with  $10^{-7}$ M DPN  $\pm$  anti-ER $\alpha$  antibody (E). PKC specific activity was measured in cell layer lysates. Values are the mean  $\pm$  SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control of each experiment.

## Discussion

The ability of E<sub>2</sub> to signal from plasma membrane receptors impacts both the genomic and nongenomic actions of the sex steroid. Estrogen activation of MAPK via src has been shown to occur in male and female osteoblasts [95]. MAPK can also be activated via PKC [96] and studies in our lab [97, 98] and others [99] suggested that the PKC signaling pathway and downstream MAPK activation [100, 101] in response to E<sub>2</sub> may be active only in cells from female animals or human donors. However, the actual receptors that mediate these responses to E<sub>2</sub> were not determined.

Our studies show that female growth plate resting zone chondrocytes respond to estrogen with an increase in both PKC and PLA<sub>2</sub> activities via ER $\alpha$  and ER $\beta$ . That the effect of E<sub>2</sub> is specific and receptor mediated was demonstrated by the fact that only E<sub>2</sub> but not Ent-E<sub>2</sub> had an effect on PKC activation. Because enantiomers are structural mirror images with identical physicochemical properties and receptors have well-defined and structurally discriminate binding pockets, they can generally identify specific ligands of different conformations. In contrast, membrane lipids present a dynamic environment that does not maintain structurally well-defined binding sites for steroids [102]. Thus, the difference in the effect of each enantiomer (E<sub>2</sub> and Ent-E<sub>2</sub>) on the cell response implies that the binding affinity of E<sub>2</sub> to the specific receptor is more effective than its enantiomer (Ent-E<sub>2</sub>). This supports our previous work showing that the stimulatory effect of E<sub>2</sub> on PKC signaling is stereospecific [61, 67].

Our results demonstrate that both ER $\alpha$  and ER $\beta$  are involved in mediating the rapid membrane-associated effects of E<sub>2</sub> on PKC and PLA<sub>2</sub> signaling pathways. Antibodies specific for ER $\alpha$  and ER $\beta$  blocked the stimulatory effects of E<sub>2</sub> on both enzymes. In addition, specific agonists for ER $\alpha$  and ER $\beta$  stimulated PKC with dose-response and time course comparable to that of E<sub>2</sub>. R,R-THC, which is an agonist for ER $\alpha$  and an antagonist for ER $\beta$  had no effect on PKC, further supporting the requirement for both receptors.

The observation that E<sub>2</sub> treatment resulted in co-immunoprecipitation of ER $\alpha$ /ER $\beta$ , suggests that a complex is formed. We did not test whether such a complex is required for membrane associated signal transduction to occur, but the fact that antibody-blocking of selective agonists for each receptor blocked the effect of E<sub>2</sub> is strong presumptive evidence that this is the case. These results support previous studies showed that ER dimerization promotes selective G-protein activation and the loss of the dimer fails to provide the structural contact points for G-proteins to bind in an active conformation, thus impairing signaling to adenylate cyclase [103]. ERs have also been shown to form a complex with androgen receptor in response to treatment with E<sub>2</sub> or dihydrotestosterone [104].

ER $\alpha$  [105] and ER $\beta$  [106], as well as truncated forms of both receptors are present in the plasma membrane [107, 108] and caveolae [109, 110], implying that membrane forms of these receptors may play a role in mediating rapid responses to the hormone. We did not address this hypothesis directly in the present study since the immunoprecipitation studies were performed using cell lysates and not isolated plasma membranes. However, our results do show that palmitoylation is required for rapid

activation of PKC by  $E_2$ , indicating that receptor recruitment to the membrane is involved [111, 112]. In contrast, inhibition of myristylation had no effect on  $E_2$ -dependent PKC activation, indicating that the ERs responsible for mediating the hormone's effect were not integral to the plasma membrane via this mechanism [113].

ERs do not display an intrinsic transmembrane domain [76]; thus an interaction of ERs with specific membrane protein has been proposed to explain their membrane localization [77, 78]. The interaction between ERs caveolin-1 and palmitoylation of ERs are the major determinants for the residence of ERs at the plasma membrane [79-81]. In this study, we found that posttranslational palmitoylation is required for ER membrane localization and for  $E_2$ -induced recruitment of ERs to the plasma membrane. It is possible that by palmitoylation, ERs are dynamically recruited from the cytoplasmic pool to the plasma membrane and most likely to the caveolae, which contains other signaling molecules including G protein  $\alpha$ -subunits, kinases, and caveolins [114]. The interaction between palmitoylated ERs and other caveolae proteins may act as a signal for the directional recruitment and restructuring of the receptor. Consequently, ERs were able to mediate further stimulation of PKC activity at 90 minutes. Thus, palmitoylation seems to be a common regulatory mechanism in the formation of membrane-associated signaling complexes [115].



## CHAPTER 3

### SEX SPECIFIC RESPONSE OF GROWTH PLATE CHONDROCYTES TO 17 $\beta$ -ESTRADIOL DIFFERENTIAL REGULATION OF PLASMA MEMBRANE ASSOCIATED ESTROGEN RECEPTOR

#### Introduction

Longitudinal growth is regulated by the activity of chondrocytes in the epiphyseal growth plates of long bones. Many hormones and growth factors are involved in the regulation of this process. Among these, sex steroids are of crucial importance, especially during puberty [116]. Estrogens are involved in the initiation of the pubertal growth spurt and in fusion of the growth plate at the end of puberty in both boys and girls. Levels of systemic estrogens are important variables, but the number and type of estrogen receptors (ERs) are also critical determinants growth plate response to the hormone [117, 118].

Both ER $\alpha$  and ER $\beta$  are present in growth plates of both males and females at the mRNA and protein level in several species, including rat, rabbit, and human [119-122], indicating that estrogens can directly regulate the tissue in addition to their indirect effects via growth hormone [123-127]. Moreover, experiments using fetal rat and neonatal mouse bone organ cultures show that they regulate growth plate development in a sex-specific manner [128]. Because the organ cultures were independent of systemic sex steroids, the results suggested that the sexual dimorphism was at the cellular level. Although the binding affinity of E<sub>2</sub> for ERs in growth plate chondrocytes was comparable in cells from both male and female rats, female cells had greater numbers of receptors than male cells [129].

While this could contribute to differences in response rate, it did not explain the marked differences in biological responses observed between male and female cells.

Whereas E<sub>2</sub> caused an increase in chondrocyte differentiation in female cells, it did not do so in male cells [130]. This effect of E<sub>2</sub> was stereospecific, indicating that it was receptor mediated. However, the mechanism involved differed from that mediating the effect of E<sub>2</sub> on chondrocyte apoptosis, which was the same in cells from both sexes [38]. Subsequent studies identified plasma membrane associated signaling in the sex-specific responses. E<sub>2</sub> caused rapid increases in Ca<sup>++</sup> ion transport [60] and activation of protein kinase C (PKC) [98] and phospholipase A<sub>2</sub> [61] in female chondrocytes but not in male cells. Importantly, these effects of E<sub>2</sub> could also be seen in cells treated with E<sub>2</sub>-conjugated to bovine serum albumin (E<sub>2</sub>-BSA), which can't pass the plasma membrane, was also able to stimulate PKC. Moreover, inhibition of PKC signaling blocked the downstream effect of E<sub>2</sub> on chondrocyte differentiation [59]. The fact that neither diethylstilbesterol nor ICI 182780, which are known to act via the nuclear ER, had an effect on the membrane associated response [59].

We hypothesized that ERs resident in the plasma membranes of growth plate chondrocytes might be responsible for the differential responses seen in male and female cells. ER $\alpha$  [131, 132] and ER $\beta$  [133-135] are the products of separate genes. In many cells, they coexist either as homodimers or as heterodimers [43, 136]. Recent evidence indicates that there are specific actions of E<sub>2</sub> that can be attributed to one receptor but not the other [137, 138]. This is supported by in vitro studies where estrogen analogs have been shown to preferentially activate the two ERs [139]. These observations, coupled with the fact that ER $\beta$  can form homodimers and heterodimers with ER $\alpha$  in vitro [43, 136], suggest the activity of estrogens may depend on whether a cell contains ER $\alpha$ , ER $\beta$ , or both.

While it is known that ER $\alpha$  and ER $\beta$  are both present in male and female cells, it is not known if they are differentially expressed or activated. It is also not known if they are transported to the membrane in a comparable manner. Of particular importance is if the membrane associated signaling pathways needed to mediate the downstream responses to E<sub>2</sub> are present in male growth plate chondrocytes. In this study, we compared the expression and subcellular localization of ER $\alpha$  and ER $\beta$  in male and female rat costochondral growth plate chondrocytes; we determined if their presence in the membrane involved palmitoylation as has been shown in other systems; and we investigated differences in the potential downstream signaling pathways.

## **Material and Methods**

### ***Reagents***

17 $\beta$ -Estradiol (E<sub>2</sub>); methyl- $\beta$ -cyclodextrin ( $\beta$ -CD), which depletes lipid rafts and alters the caveolar microenvironment [72, 140]; the phospholipase C (PLC) inhibitor U73122 [141]; and the PLA<sub>2</sub> inhibitor AACOCF<sub>3</sub> [142] were purchased from Sigma Chemical Co. (St. Louis, MO). The ER $\alpha$ -selective agonist propyl pyrazole triol (PPT) [143-145] ; the PLC agonist *m*3M3F $\beta$ S [146] ; and the ER $\beta$ -selective agonist diarylpropionitrile (DPN) [143-145] were obtained from Tocris Cookson Inc. (Ellisville, MO). Tunicamycin, an inhibitor of palmitoylacyltransferase [113], was purchased from EMD Chemicals, Inc. (San Diego, CA). ER $\alpha$  monoclonal antibody (ab16460) and ER $\beta$  monoclonal antibody (ab16813) were purchased from Abcam (San Francisco, CA). A polyclonal antibody to caveolin-1 (Cav-1) was purchased from Santa Cruz Biotechnology (sc-894, Santa Cruz, CA). PKC BioTrak assay kits were obtained from GE Lifesciences (Piscataway, NJ) and Dulbecco's modified Eagle medium (DMEM) was obtained from

GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay kit [87] obtained from Pierce Chemical Co. (Rockford, IL). [<sup>32</sup>P]-ATP was obtained from Perkin Elmer (Melville, NY).

### ***Chondrocyte Cultures***

The culture system used in this study has been described in detail previously [147]. Chondrocytes were isolated from the resting zone cartilage of the costochondral junction of 150 gram male and female Sprague-Dawley rats, plated at an initial density of 10,000 cells/ cm<sup>2</sup>, and maintained in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml ascorbic acid. The medium was replaced after 24 hours and then every 48 hours until cultures reached confluence. Fourth passage cells were used for all experiments based on previous studies showing that these cells preserve their chondrocyte phenotype as well as their differential responsiveness to vitamin D metabolites at this passage [89]. Confluent fourth passage male and female cells in 24 well plates were treated for 90 minutes with 0.5 ml of ethanol at the highest concentration used in experimental cultures or experimental media (DMEM + 10% FBS + appropriate concentration of E<sub>2</sub>).

### ***ER Expression and Subcellular Localization***

#### ***Plasma Membrane and Caveolae Isolation***

Plasma membranes were isolated using a detergent-free method as described previously [148]. Confluent, fourth passage resting zone chondrocytes isolated from female and male rats were treated with either 10<sup>-7</sup> M E<sub>2</sub> or vehicle for 90 minutes. The cell layers were washed two times with 1XPBS to remove any residual medium and cells were harvested by scraping using isolation buffer (0.25 M sucrose, 1 mM EDTA, 20 mM

Tricine, pH 7.8). Cells were homogenized using a tissue grinder and the homogenate centrifuged at 3500rpm for 10 minutes to pellet large debris including the nucleus, mitochondria, and endoplasmic reticulum. The supernatant was collected and layered on 30% Percoll in isolation buffer (GE Healthcare, Piscataway, NJ). Plasma membranes were collected from the samples by centrifuging at 30,500 rpm for 30 min and removing the visible band in the middle of the gradient column. The plasma membrane fraction was then layered over a 10–20% OptiPrep gradient (Sigma–Aldrich), and the gradient was centrifuged at 26,500 rpm for another 4 hours. Plasma membrane sub-fractions were collected from the top to the bottom of the tube, which resulted in isolation of 15 fractions. Caveolae were observed as an opaque band, which was collected in fraction 3.

Western blots were used to determine if ER $\alpha$  and/or ER $\beta$  were present in whole cell lysates and the distribution of the ER isoforms in plasma membranes and caveolae in the male and female cells. Plasma membranes, the caveolae fraction and the nuclear fraction were separated on 4–20% gradient acrylamide gels. Blots of the gels were probed with either the mouse monoclonal antibody against ER $\alpha$  or the mouse monoclonal antibody against ER $\beta$ . In addition, blots were probed using a polyclonal antibody to caveolin-1 (Cav-1). Immunoreactive bands were visualized using goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). The Super Signal West Pico Chemiluminescent System (Thermo Fisher Scientific) was used to treat the membrane and then imaged with the VersaDoc imaging system (Bio-Rad).

#### *Palmitoylation*

To assess the role palmitoylation in localizing ER $\alpha$  and ER $\beta$  to the plasma membrane as well as the role of E<sub>2</sub> in regulating ER $\alpha$  and ER $\beta$  palmitoylation, male and

female cultures were pre-incubated with 30 $\mu$ M tunicamycin for 2 hours. The cells were then treated with 10<sup>-7</sup> M E<sub>2</sub> for 90 minutes. Plasma membranes were isolated and analyzed as above.

#### *Receptor Complex Formation*

To determine if the ER isoforms formed complexes in response to E<sub>2</sub>, fourth passage chondrocytes from female rats were treated  $\pm 10^{-7}$  M E<sub>2</sub> for 90 min. At harvest the cell layers were washed with 1XPBS and the cells were lysed and sonicated in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM disodium EDTA, 1% Nonidet P-40) containing 100 mM NaF, protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein samples were mixed with anti-ER $\alpha$  antibody and incubated at 4°C overnight with continuous agitation. Immunoprecipitation was measured in protein samples using a commercially available Dynabeads® Protein kit following the manufacturer's directions (Invitrogen, Grand Island, NY). In order to lower the background in the images, a ONE-HOUR IP-Western Kit (Genscript) was used. The immunoprecipitated samples were then subjected to Western blot analysis.

#### *Receptor Number*

The number of the ERs on the plasma membrane and intracellularly were evaluated by flow cytometry using the Quantum Simply Cellular Microbeads Kit (Bangs Laboratories, Inc. Fishers, IN). This kit provides a method for the evaluation of the number of molecules of antibody per cell. Briefly, this kit is comprised of populations of uniform cell-sized microspheres with different calibrated binding capacities of goat anti-mouse IgG (FC-specific) on their surface. The kit includes 4 coated populations, each with different antibody binding capacity (ABC) for mouse monoclonal antibodies, and 1

blank population with no specific binding capacity for mouse IgG. When the bead populations are labeled in the same manner as the cells to be analyzed, they provide a means of constructing a QuickCal<sup>®</sup> calibration curve (ABC values versus fluorescence intensity), from which samples may be analyzed.

The number of ER $\alpha$  cell surface receptors was determined by incubating male and female cell suspensions ( $5 \times 10^5$  cells) for 30 minutes in 150 $\mu$ l 1% BSA with a 1:50 dilution of the ER $\alpha$  monoclonal antibody. Cells were then washed with 1 ml 1% BSA, centrifuged for 5 minutes at 1000 rpm, and resuspended in 150 $\mu$ l 1% BSA containing a 1:500 dilution of goat-anti mouse antibody conjugated to Alexa Fluor<sup>®</sup> (Invitrogen, Grand Island, NY) for 30 minutes in the dark. Cells were centrifuged at 1000 rpm for 10 minutes and resuspended in 300 $\mu$ l PBS. After collecting the cells by filtration, samples were ready for analysis.

To determine the total number of receptors (surface and cytoplasmic), cells were fixed by incubating with 4% paraformaldehyde in 1XPBS for 20 minutes at 4°C. Then cells were collected by centrifugation at 1000 rpm for 10 minutes and resuspended in permeabilization solution (0.5% Triton X-100 in PBS) at room temperature for 15 minutes. Permeabilized cells were incubated with the primary antibody and further processed as described above. Microspheres (50 $\mu$ L per tube) were incubated with the secondary antibody, washed and further processed on the flow cytometer under the same conditions as the samples.

## ***Signaling Pathways***

### ***Protein Kinase C Activity***

Confluent fourth passage male and female cells in 24 well plates were treated for 90 minutes with 0.5 ml of ethanol at the highest concentration used in experimental cultures or experimental media (DMEM + 10% FBS +  $10^{-7}$  to  $10^{-10}$  M  $E_2$ ). To determine which ERs were involved in this pathway, male and female cultures were treated for 90 minutes with  $10^{-7}$  to  $10^{-9}$  M ER $\alpha$ -selective agonist PPT or ER $\beta$ -selective agonist DPN, respectively. To determine if the increase in PKC was caveolae dependent, female cultures were pre-incubated for 30 minutes with 5mM methyl- $\beta$ -cyclodextrin ( $\beta$ -CD)[140], and then treated for 90 minutes with  $10^{-7}$  M  $E_2$ .

We previously showed that phosphatidylinositol-specific phospholipase C (PLC) mediated the rapid increase in  $E_2$ -dependent PKC [59]. To establish if PLC also plays a role in the increase in PKC at 90 minutes, confluent cultures of female cells were treated with  $E_2 \pm$  the PLC inhibitor U73122. To determine if the downstream signaling pathway was functional in male cells, male and female cells were treated with PLC activator *m*-3M3F $\beta$ S and PKC activity was measured. After the incubation period, cell layers were washed with 1XPBS to remove any excess medium and then lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluide and 1% NP-40). 25 $\mu$ l of the cell lysates were assayed for protein content [87]. PKC activity was measured in cell lysates using a kit following the manufacturer's directions (Amersham Bioscience). This kit is optimized for measurement of phospholipid and  $Ca^{2+}$ -dependent PKC [149, 150].



### *Regulation of PGE<sub>2</sub> Release*

PGE<sub>2</sub> production is a downstream consequence of PLA<sub>2</sub> activation and can activate the protein kinase C (PKC) signaling pathway leading to ERK1/2 activation [39]. To determine if PGE<sub>2</sub> release into the medium is produced in response to E<sub>2</sub>, female cells were pre-incubated for 30 minutes with 10<sup>-6</sup>–10<sup>-5</sup> M of the PLA<sub>2</sub> inhibitor AACOCF<sub>3</sub>, and then cells were treated with 10<sup>-7</sup> M E<sub>2</sub> for 90 minutes. Male and female cells were then treated for 30 minutes with 10<sup>-6</sup>-10<sup>-5</sup> M PLAA peptide, which activates PLA<sub>2</sub> [151]. At the end of the incubation, the media were acidified and PGE<sub>2</sub> was measured using a commercially available kit (Prostaglandin E<sub>2</sub> [<sup>125</sup>I]-RIA kit, NEK020001 K, Perkin Elmer, Waltham, MA). PGE<sub>2</sub> data were normalized to total DNA (Quant-iT™ PicoGreen® dsDNA Assay kit, P11496, Invitrogen).

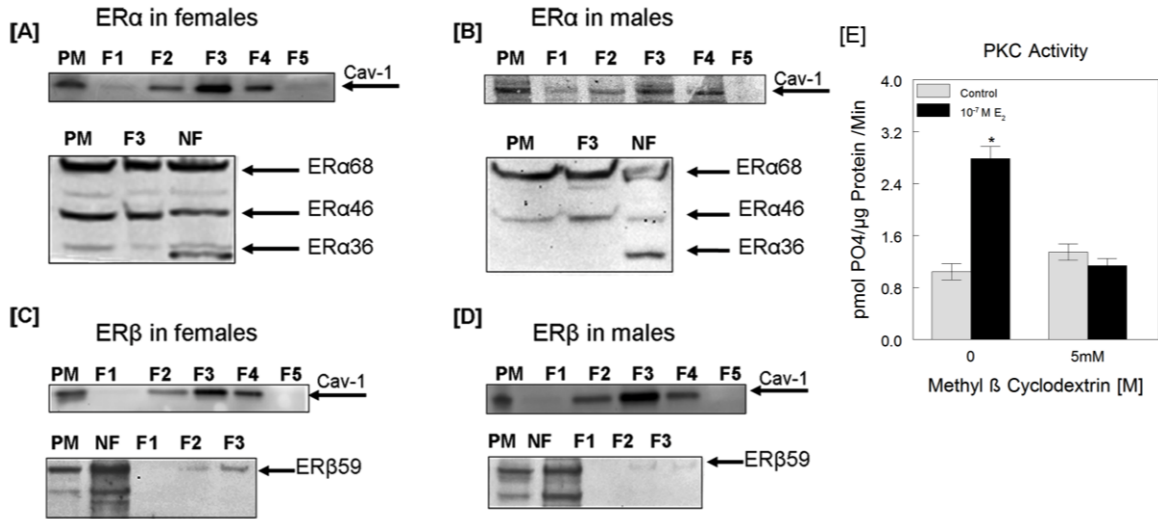
### *Statistical Management of the Data*

For each experiment, each value represents the mean ± SEM of the cell layers of six independent cultures. Statistical significance was determined by ANOVA. P<0.05 was considered significant. Each experiment was repeated two or more times to ensure the validity of the data. The data represented are from a single representative experiment.

## **Results**

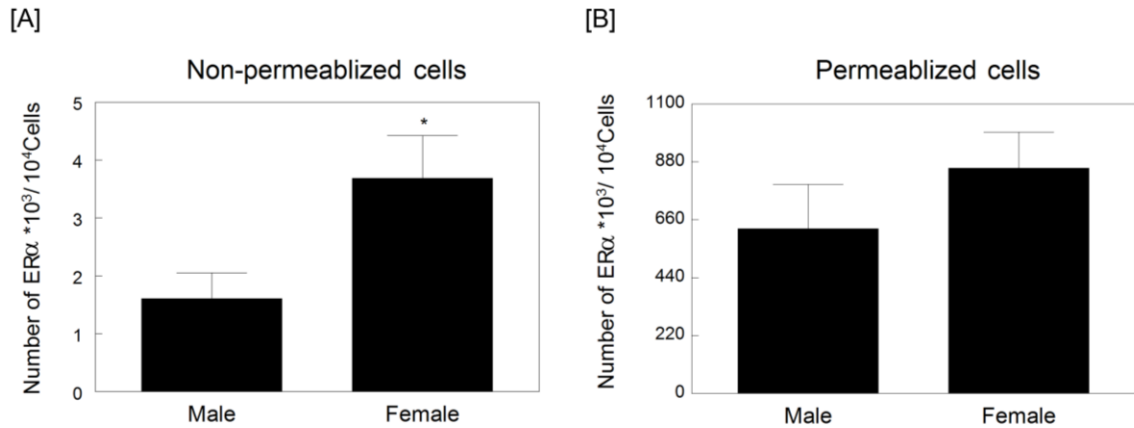
Western blots of the cell lysates demonstrated that ERα was present in female and male resting zone chondrocytes (data not shown). The nuclear fraction from both cell types exhibited all three ERα isoforms: ERα68, ERα46 and ERα36 (Figure 3.1A,B). Plasma membranes from female chondrocytes had all three isoforms, but male cells lacked ERα36. This was also the case for caveolae. ERβ59 was present in plasma membranes and nuclear fraction from female and male cells and was present in caveolae

from female cells but not male cells (Figure 3.1C,D). Intact caveolae were required  $E_2$  activation of PKC in female cells. Treatment with methyl  $\beta$ -cyclodextrin blocked the stimulatory effect of the hormone (Figure 3.1 E)



**Figure 3.1: Difference in the amount and subcellular localization of ERα and ERβ between male and female chondrocytes.** Subcellular localization of ERα in female resting zone chondrocytes (A), or male resting zone chondrocytes (B). Subcellular localization of ERβ in female resting zone chondrocytes (C), or male resting zone chondrocytes (D). WCL Whole cell lysates, PM plasma membrane, F3 of caveolae fraction, and NF nuclear fraction were isolated and subjected to western blot. Female cultures were pre-incubated for 30 minutes with 5mM methyl-β-cyclodextrin (β-CD) then cells were treated with  $10^{-7}$  M  $E_2$  for 90 minutes. PKC specific activity was measured in cell layer lysates. Values are the mean± SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control, ^P<0.05,  $E_2$ + β-CD vs.  $E_2$  alone.

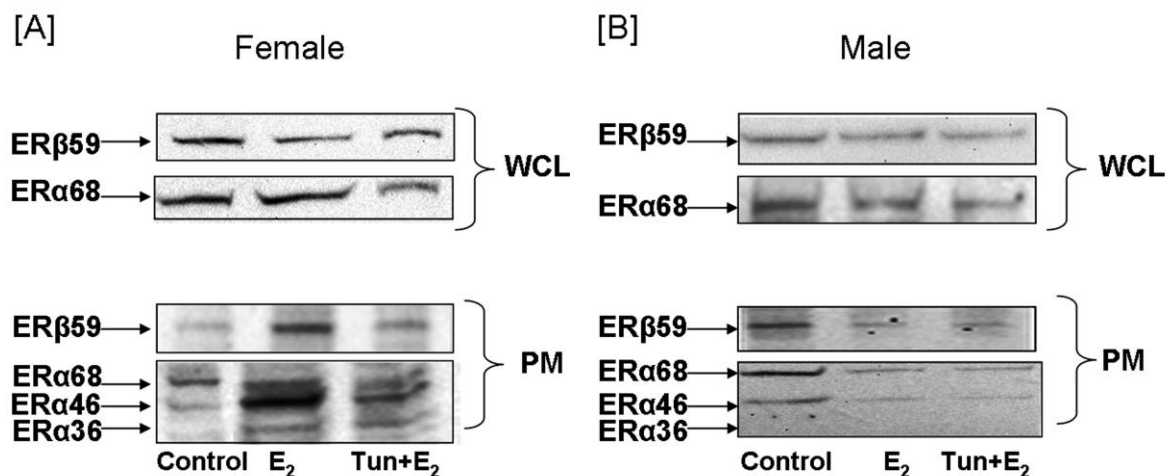
Sex-specific differences in the amount and distribution of ER $\alpha$  were confirmed by flow cytometry. There was more than twice as much plasma membrane ER $\alpha$  in non-permeabilized female cells than in male cells. Total ER $\alpha$  in permeabilized cells was not statistically different, however (Figure 3.2 A,B). Interestingly, the percentage of positive cells was not significantly different in both permeabilized and non-permeabilized cells female and male cells.



**Figure 3.2: Difference in the number of ER $\alpha$  between male and female chondrocytes.** Flow cytometry result showing the number of the plasma membrane ER $\alpha$  in non permeabilization cells (A), and the number of total ER $\alpha$  (plasma membrane and nuclear receptors) in permeabilization cells (B). Values are the mean  $\pm$  SEM for six independent cultures for each variable. \*P<0.05, female vs. male.

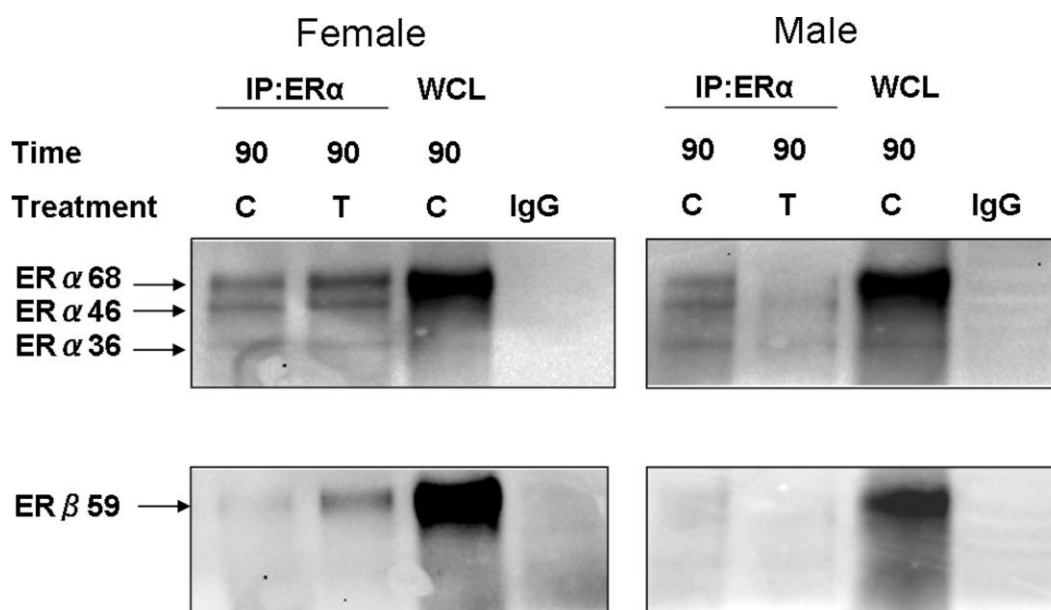
Tunicamycin reduced the amount of ER $\alpha$ 68 in whole cell lysates of female chondrocytes but had no effect on the male cells (Figure 3.3 A,B). E<sub>2</sub> caused an increase in plasma membrane ER $\alpha$ 46 and ER $\alpha$ 36, as well as ER $\beta$ 59 in female cells, all of which were reduced by pretreatment with tunicamycin.

### Effect of $E_2 \pm$ Tunicamycin on $ER\alpha$ and $ER\beta$ in Chondrocytes



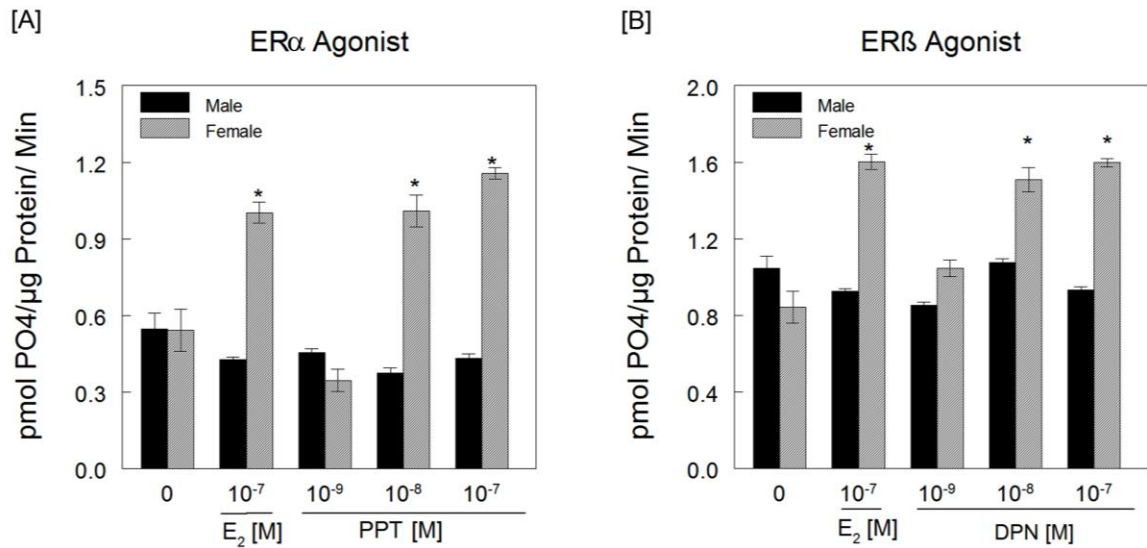
**Figure 3.3: Palmitoylation of the membrane receptor is required for  $E_2$ -dependent PKC activity only in female chondrocytes.** Female cells were either treated for 90 minutes with  $10^{-7}$  M  $E_2$  alone or with 30 $\mu$ M tunicamycin for 2 hours then cells were treated with  $10^{-7}$  M  $E_2$  for 90 min. Presence of ER $\alpha$  and ER $\beta$  in female chondrocytes (A) or male chondrocytes (B) in whole cell lysates and plasma membrane was examined by western blot.

These observations were confirmed by co-immunoprecipitation of ER $\alpha$ . Treatment of female chondrocytes for 90 minutes with  $E_2$  increased the amount of ER $\alpha$ 68, ER $\alpha$ 46 and ER $\beta$ 59 that was present in the immunoprecipitates, but in male cells, the levels of all three isoforms were reduced (Figure 3.4).



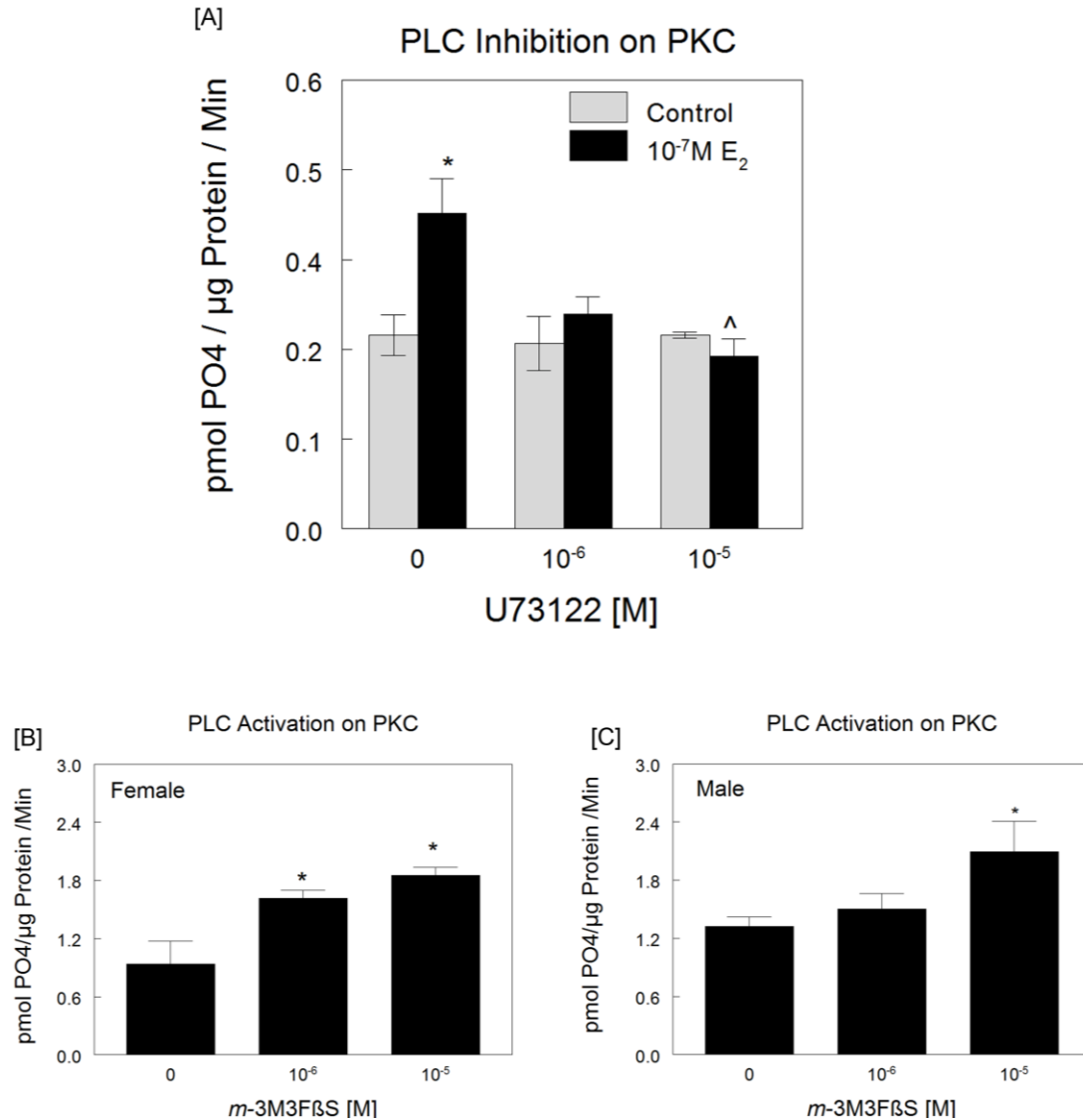
**Figure 3.4. ER $\alpha$  was immunoprecipitated and subjected to Western blot.** The membranes were incubated with anti-ER $\alpha$  and anti-ER $\beta$  antibodies. C: control group, T: treated group.

Both ER $\alpha$  and ER $\beta$  were involved in the E<sub>2</sub>-dependent activation of PKC in female cells. Treatment of the cells with the ER $\alpha$  agonist PPT (Figure 3.5A) or the ER $\beta$  agonist DPN (Figure 3.5B) had the same stimulatory effect on PKC as E<sub>2</sub>. In contrast, activation of ER $\alpha$  or ER $\beta$  using the respective agonists had no effect on PKC in male cells.



**Figure 3.5: Dose-dependent effect of PPT and DPN on PKC specific activity on resting zone chondrocytes.** Confluent fourth passage female and male chondrocytes were treated for 90 minutes with  $10^{-7}$  to  $10^{-9}$  M PPT (A) or  $10^{-7}$  to  $10^{-9}$  M DPN (B). PKC specific activity was measured in cell layer lysates. Values are the mean  $\pm$  SEM for six independent cultures for each variable. \* $P < 0.05$ , treatment vs. control at each time point.

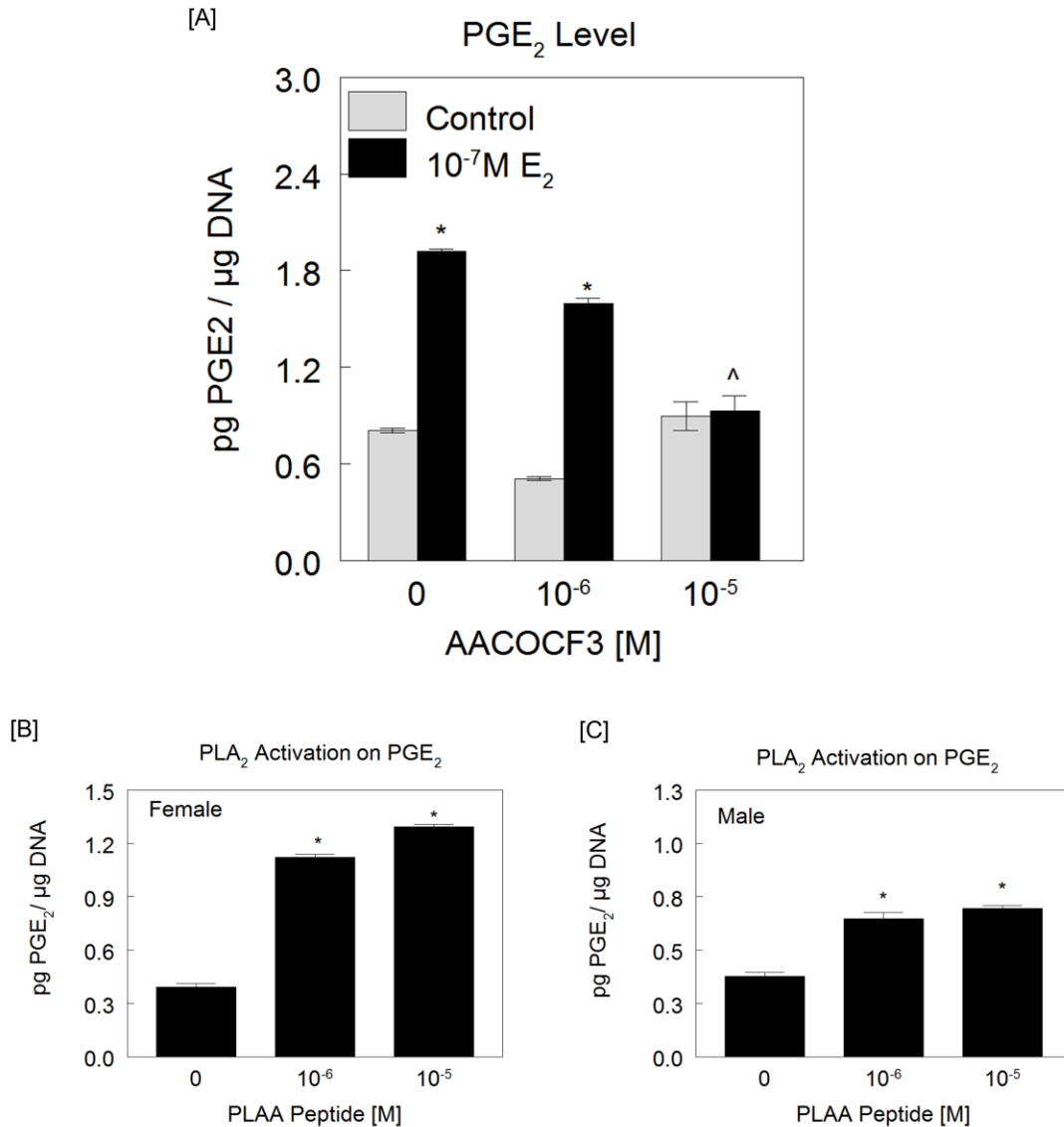
Inhibition of PLC blocked the stimulatory effect of E<sub>2</sub> on PKC in the female cells, even at 90 minutes (Figure 3.6A). Conversely, the PLC activator *m*-3M3F $\beta$ S induced an increase in PKC activity in the female cells (Figure 3.6B). Male cells also responded to the PLC activator with an increase in PKC, but the effect was seen only at the highest concentration (Figure 3.6C).



**Figure 3.6: Activation of PKC by E<sub>2</sub> is dependent on PLC signaling.** Confluent, fourth passage resting zone cells from female rat (A,B) or male rat (C) were treated for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> ± 10<sup>-6</sup>-10<sup>-5</sup> M PLC inhibitor U73122 (A) or 10<sup>-6</sup>-10<sup>-5</sup> M PLC activator *m*-3M3FβS (B,C). \*P<0.05, treatment vs. control, ^P<0.05, E<sub>2</sub> + U73122 vs E<sub>2</sub> alone at a particular concentration of 17β-estradiol.

PGE<sub>2</sub> production in response to E<sub>2</sub> was blocked by the PLA<sub>2</sub> inhibitor AACOCF<sub>3</sub> (Figure 3.7A). Activation of PLA<sub>2</sub> with PLAA also caused an increase in PGE<sub>2</sub> release in the female cells (Fig. 3.7B). Male cells exhibited an increase in PGE<sub>2</sub> release when

treated with PLAA, but the stimulatory effect was less robust than was observed in the female cells (Figure 3.7C).



**Figure 3.7: Production of PGE<sub>2</sub> by E<sub>2</sub> is dependent on PLA<sub>2</sub> signaling.** Confluent, fourth passage resting zone cells from female rat (A,B) or male rat (c) were treated for 90 minutes with 10<sup>-7</sup> M E<sub>2</sub> ± 10<sup>-6</sup>-10<sup>-5</sup> M Cytosolic PLA<sub>2</sub> inhibitor ACCOCF3 (A) or 10<sup>-6</sup>-10<sup>-5</sup> M PLA<sub>2</sub> activator PLAA peptide (B,C). \*P<0.05, treatment vs. control, ^P<0.05, E<sub>2</sub> + ACCOCF3 vs. E<sub>2</sub> alone at a particular concentration of E<sub>2</sub>.



## Discussion

This study shows that PKC activity is regulated by E<sub>2</sub> in growth plate chondrocytes in a sex-specific manner. Only female resting zone chondrocytes exhibited an increase in PKC activity when treated with E<sub>2</sub>. As noted previously, the effects of E<sub>2</sub> on PKC are membrane-dependent based on the observation that E<sub>2</sub>-BSA elicits a comparable response. E<sub>2</sub>-BSA has been shown to bind to surface receptors for E<sub>2</sub> [73], and remain extracellular [152]. Further support that specific receptors are involved is the observation that the activation of PKC is stereospecific; only treatment with 17 $\beta$ -estradiol but not 17 $\alpha$ -estradiol resulted in increased enzyme activity. Only E<sub>2</sub> but not *ent*-E<sub>2</sub> had an effect on PKC activation, this strongly suggests that the effects of E<sub>2</sub> are direct and specific to a membrane receptor. Thus, the difference in the effect of each enantiomer (E<sub>2</sub> and *ent*-E<sub>2</sub>) on the cell response implies that the binding affinity of E<sub>2</sub> to the specific receptor is more effective than its enantiomer (*ent*-E<sub>2</sub>).

Our findings are commensurate with those in other laboratories demonstrating that E<sub>2</sub> acts via membrane receptors, including ER $\alpha$  and ER $\beta$  [70, 108, 113]. Both ER $\beta$  and ER $\alpha$  selective agonists DPN and PPT activate PKC suggesting that both receptors are involved. The fact that ER $\alpha$  [70, 105] and ER $\beta$  [106], as well as truncated forms of both receptor are present in the plasma membrane [107, 108] and caveolae [109, 110] implies that membrane forms of these receptors may play a role.

In this study we found that E<sub>2</sub>-induced recruitment of ERs to the plasma membrane only in female chondrocytes. The ability of tunicamycin an inhibitor of palmitoyltransferase to inhibit the dynamically recruited of ERs from the cytoplasmic pool to the plasma membrane and most likely to the caveolae, suggests that

posttranslational palmitoylation is required for ERs membrane localization and for E<sub>2</sub>-induced recruitment of ERs to the plasma membrane. While in male cells E<sub>2</sub> treatment decreased the translocation of ERs to the plasma membrane. Furthermore, tunicamycin did have any effect.

This result suggest that, It is possible that by palmitoylation in female chondrocytes , ERs are dynamically recruited from the cytoplasmic pool to the plasma membrane and most likely to the caveolae, which contains other signaling molecules including G protein  $\alpha$ -subunits, kinases, and caveolins [114, 153]. The interaction between palmitoylated ERs and other caveolae proteins may act as a signal for the directional recruitment and restructuring of the receptor. Consequently, ERs were able to mediate further stimulation of PKC activity at 90 minutes. Thus, palmitoylation seems to be a common regulatory mechanism in the formation of membrane-associated signaling complexes [115].

The ability of downstream PLC activators *m*-3M3FBS to induce an increase in PKC activity in male and female cells, similarly, PLAP peptide downstream PLA<sub>2</sub> activators to induce increase in PGE<sub>2</sub> level in both male and female cells, suggests, although female chondrocytes expressed more ER $\alpha$  and ER $\beta$  on the membrane and caveolae fraction than male chondrocytes, they shared similar signaling pathways downstream of the receptors. Palmitoylation of the membrane receptor might contribute to their gender-specific membrane-mediated responses to E<sub>2</sub>.

## **CHAPTER 4**

### **RAPID MEMBRANE RESPONSES TO DIHYDROTESTOSTERONE ARE SEX DEPENDENT IN GROWTH PLATE CHONDROCYTES**

#### **Introduction**

Sex steroid hormones have key effects on the development and maintenance of the skeleton and its sexual dimorphism [154, 155]. The principle steroidal androgens, testosterone (T) and its metabolite 5 $\alpha$ -dihydrotestosterone (DHT) play an important role in skeletal development in males during adolescence [156]. In certain target tissues such as the growth plate, liver, brain, prostate and skin, testosterone is irreversibly converted to DHT by the enzyme 5 $\alpha$ -reductase [157]. DHT is considered to be the more potent hormone because it has a higher androgen receptor binding affinity and a lower dissociation constant than testosterone [158]. Androgen deficiency results in various abnormalities of bone metabolism, including decreased bone mineral density as well as a lower bone mass and accelerated bone loss due to increased bone resorption, leading to a higher fracture risk [154]. It was thought that these effects were mainly caused by classic nuclear receptors, which act as transcription factors, thereby modulating gene expression [159]. However, recent studies indicate that rapid alternative pathways are also involved [160, 161].

These rapid responses to androgens occur within seconds to minutes after administration of the hormone and are generally insensitive to inhibitors of the androgen receptor (AR) [162], suggesting that they are regulated by cellular signaling pathways involving surface membrane receptors and second messengers [163]. This mechanism of steroid action has been referred to as non-genomic, although they can result in changes in downstream gene expression [17]. In cultured cardiac myocytes, testosterone has been

found to induce a rapid increase in intracellular  $\text{Ca}^{2+}$  through activation of a plasma membrane androgen receptor associated with the pertussis toxin (PTX)-sensitive G protein-coupled phospholipase C (PLC), inositol triphosphate signaling pathway [21, 22]. Moreover treatment of LNCaP prostate cells with testosterone coupled to bovine serum albumin (T-BSA), which cannot cross the membrane has been shown to cause phosphorylation and activation of focal adhesion kinase and increase secretion of prostate-specific antigen within minutes [164]. The ability of androgens to rapidly modulate the activity of ion channels and  $\text{Ca}^{2+}$  has been observed in several cell types including endothelial cells [165], breast cancer cells [166], prostate cancer cells [166, 167], macrophages [168], and T-lymphocytes [169, 170]. Of note, the ability of testosterone or T-BSA to increase  $\text{Ca}^{2+}$  in primary osteoblasts was found to be sexually dimorphic [24]. Testosterone was only able to induce  $\text{Ca}^{2+}$  influx in male primary osteoblasts but not those cells derived from females. In contrast,  $\text{Ca}^{2+}$  influx in female osteoblasts was affected by estradiol, in a non-genomic fashion, whereas male osteoblasts were estradiol insensitive.

In long bones, endochondral bone formation occurs at the growth plate, a region of developing cartilage located between the epiphysis and the metaphysis [171]. The process of endochondral ossification is regulated in part by sex steroid hormones [27]. Androgens stimulate endochondral bone growth and elongation [28] while estrogen is known to suppress longitudinal bone growth and accelerate growth plate closure [29, 33, 34, 75, 172-175]. Studies using rat growth plate chondrocytes as a model show that the effects of  $17\beta$ -estradiol (E2) on apoptosis are found in both male and female cells and the same mechanism is involved [176]. However, growth plate chondrocytes are also

regulated by sex steroids in a sexually dimorphic manner [75]. E2 causes a rapid activation of  $\text{Ca}^{2+}$  influx and activation of PKC only in cells from female rats [75, 174], but it is not known if testosterone or DHT has a similar effect on cells from male rats.

The goal of the present study was to assess testosterone and / or DHT-dependent rapid responses in growth plate chondrocytes and determine the mechanisms involved. To do this, we took advantage of the rat costochondral cartilage chondrocyte model in which cells at distinct states of endochondral development can be isolated and cultured in vitro up to four passages without loss of phenotype [177]. Chondrocytes from the resting zone of the growth plate were treated with testosterone and DHT, and rapid effects on phospholipase A2 (PLA2) and PKC signaling pathways determined.

## **Materials and Methods**

### ***Reagents***

5 $\alpha$ -Dihydrotestosterone (DHT); testosterone; G-protein inhibitor pertussis toxin (PTX, G $\alpha$ i inhibitor) [178], cholera toxin (CTX, G $\alpha$ s activator) [179], and GDP $\beta$ s (general G-protein inhibitor) [180, 181]; and PLA2 inhibitors AACOCF3 [182] and quinacrine [183] were obtained from Sigma Chemical Co. (St. Louis, MO). Thapsigargin as an inhibitor of a Ca-ATPase pump in the endoplasmic reticulum [184], and verapamil hydrochloride as well as nifedipine as inhibitors of specific L type  $\text{Ca}^{2+}$  channels on the cell membrane [185, 186], actinomycin D [187] and the translation inhibitor cycloheximide [188, 189] were purchased from Sigma Chemical Co. (St. Louis, MO). U73122 (phospholipase C inhibitor) [190] was obtained from Cayman Chemical (Ann Arbor, MI). A PKC assay kit was obtained from Amersham Bioscience (Piscataway, NJ) and Dulbecco's modified Eagle medium (DMEM) was obtained from GIBCO-BRL

(Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay kit [87] obtained from Pierce Chemical Co. (Rockford, IL). [ $^{32}$ P]-ATP and [ $^3$ H]-thymidine were obtained from Perkin Elmer (Melville, NY).

### ***Chondrocyte Cultures***

The culture system used in this study has been described in detail previously [88]. Chondrocytes were isolated from the resting zone of costochondral junction of 150 g male and female Sprague-Dawley rats. To limit the contamination by fibroblasts and osteoblasts, perichondrium tissue and calcified cartilage were discarded. After dissection, the cartilage was sliced and incubated overnight in DMEM in a 5% CO<sub>2</sub> atmosphere and 100% humidity at 37°C for 24h. Chondrocytes were released from the tissue by sequential incubations in 1% trypsin for 1 h and in 0.02% Type II collagenase for 3 h in Hanks' balanced salt solution. Chondrocytes were plated at an initial density of 10,000 cells/cm<sup>2</sup>. Cells were incubated in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml vitamin C in atmosphere of 5% CO<sub>2</sub> and 100% humidity at 37°C. The culture media were replaced after 24 h and then at 72 h until cells reached confluence. Fourth passage cells were used for all experiments based on previous studies showing that these cells preserve their chondrogenic phenotype as well as their differential responsiveness to vitamin D metabolites at this passage [89].

### ***Protein Kinase C***

In order to determine if testosterone or DHT cause rapid activation of PKC, confluent fourth passage male and female cells in 24 well plates were treated for different time periods with 0.5 ml of ethanol at highest concentration used in experimental cultures

or experimental media (DMEM + 10% FBS + various concentrations of testosterone or DHT). Experimental time points 9, 90 and 270 min were examined. After the incubation period, cell layers were washed with PBS to remove any excess media and then lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride and 1% NP-40). 25 µl of the cell lysates were assayed for protein content [87]. PKC activity was measured in cell lysates using a kit following the manufacturer's directions (Amersham Bioscience). This kit is optimized for measurement of phospholipid and  $\text{Ca}^{2+}$ -dependent PKC [92, 191].

### ***Mechanism of PKC Activation***

#### ***Phospholipase A2***

To assess the effects of testosterone and DHT on PLA2 activity, the following protocol was used. For each experiment, confluent cultures of male chondrocytes in 24 well plates were treated for 9 minutes with 0.5 ml of the control media (0.02% ethanol in DMEM + FBS) or experimental media (DMEM + 10% FBS + various concentrations of testosterone or DHT). After the treatment period, cell layers were washed with phosphate buffered saline (PBS) and then cells were sonicated in 0.1 ml of cold buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid containing 1 mM EDTA). Cell pellets were obtained by centrifugation for 15 minutes. 25 µl of the cell lysates were assayed for protein content [87]. PLA2 activity was measured in cell supernatant using a kit following the manufacturer's directions (VWR). Samples were analyzed using a plate reader at 414 nm [93, 94, 192]. To verify the role of PLA2, the secretory PLA2 inhibitor quinacrine [183] or the cytosolic PLA2 inhibitor AACOCF3 [182] were used to inhibit PLA2 activity. Cultures were incubated for 9 minutes with control media or  $10^{-7}$  M DHT

in the presence or absence of 0.1 or 1 or 10  $\mu\text{M}$  quinacrine or AACOCF<sub>3</sub> and assayed for PKC activity.

#### *Phospholipase C*

Involvement of PLC was examined using U73122, an inhibitor of phosphatidylinositol (PI)-specific PLC [190]. Cultures were incubated for 9 minutes with the control media or  $10^{-7}$  M DHT in the presence or absence of 0.1, 1 or 10  $\mu\text{M}$  U73122, and PKC specific activity was determined [87, 89].

#### *G-Proteins*

To examine the role of G-proteins, the nonhydrolyzable GDP $\beta$ s [180, 181] (general G-protein inhibitor), cholera toxin (CTX, G $\alpha$ s activator) [179, 193] and pertussis toxin (PTX, G $\alpha$ i inhibitor) [15] were used. Cultures were treated for 9 minutes with control media or  $10^{-7}$  M DHT in the presence or absence of 1 or 10  $\mu\text{M}$  PTX or CTX. In other experiments, cultures were treated for 9 minutes with control media,  $10^{-7}$  M or  $10^{-8}$  M DHT in the presence or absence of 0.1  $\mu\text{M}$  GDP $\beta$ s.

#### *Intracellular Ca<sup>2+</sup>*

In order to determine the source of the intracellular calcium involved in the non-genomic mechanisms in PKC activation, we used three different inhibitors. Thapsigargin is an inhibitor of a Ca-ATPase pump in the endoplasmic reticulum [184]. The cultures were incubated for 30 min with 0.1 or 1  $\mu\text{M}$  thapsigargin prior to treatment with DHT in order to deplete internal stores of Ca<sup>2+</sup>.  $10^{-7}$  M DHT was then added for 9 minutes and the cultures lysed and assayed for PKC activity. The same strategy was used with two inhibitors of L type Ca<sup>2+</sup> channels on the cell membrane: verapamil and nifedipine [185,



186]. Cultures were pretreated with 1 or 10  $\mu\text{M}$  verapamil or nifedipine for 30 minutes and then with DHT for 9 minutes. Cell layer lysates were assayed for PKC activity.

#### *Androgen Receptor*

The role of the classical androgen receptor in the effect of DHT on PKC was examined using cyproterone acetate (CPA) to inhibit receptor binding [194]. Male chondrocytes were treated for 9 minutes with control media or  $10^{-8}$ - $10^{-7}$  M DHT in the presence or absence of 0.1  $\mu\text{M}$  CPA.

#### *Requirement for Transcription and Translation*

To examine the involvement of genomic mechanisms in PKC activation, the chondrocyte cultures were treated with DHT in the presence of the transcription inhibitor actinomycin D [187] or the translation inhibitor cycloheximide [188, 189]. Cultures were incubated with  $10^{-7}$  M DHT and 0.1 mM actinomycin D or 0.1 mM cycloheximide for 9 min. After treatment with the inhibitors, the cells were washed with PBS and assayed for PKC activity as described above.

#### ***Role of PKC in Mediating the Physiologic Response to DHT***

##### *[3H]-Thymidine Incorporation*

DNA synthesis was tested by measuring [ $^3\text{H}$ ]-thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously [89]. Cultures of male resting zone chondrocytes were incubated for 48 h with DMEM containing 1% FBS in order to achieve quiescence. The media were replaced with control media or  $10^{-7}$  M DHT for 9 minutes, at which time the media were replaced with DMEM containing 1% FBS for 24 h. Four hours prior to harvest, [ $^3\text{H}$ ]-thymidine (2  $\mu\text{Ci/ml}$ ) was added. At harvest, the cell layers were washed twice with cold PBS and twice with TCA,

then treated with saturated TCA for 30 min. TCA-perceptible material was dissolved in 0.2 ml 1% sodium dodecyl sulfate, and radioactivity was measured by liquid scintillation spectroscopy.

#### *Alkaline Phosphatase Specific Activity*

To examine the effect of DHT on alkaline phosphatase activity, confluent cells were treated with medium containing vehicle alone or  $10^{-9}$ - $10^{-7}$  M DHT for 9 minutes. After 9 minutes, the media were replaced with DMEM containing 1% FBS for 24 hours. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline] specific activity was measured in the cell layers lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2 [195] and normalized to protein content [87].

#### *Phospholipase C*

The involvement of PLC was examined using U73122. Cultures were incubated for 9 minutes with control media or  $10^{-7}$  M DHT in the presence or absence of 0.1  $\mu$ M U73122. Alkaline phosphatase was assayed at 24 hours as described above.

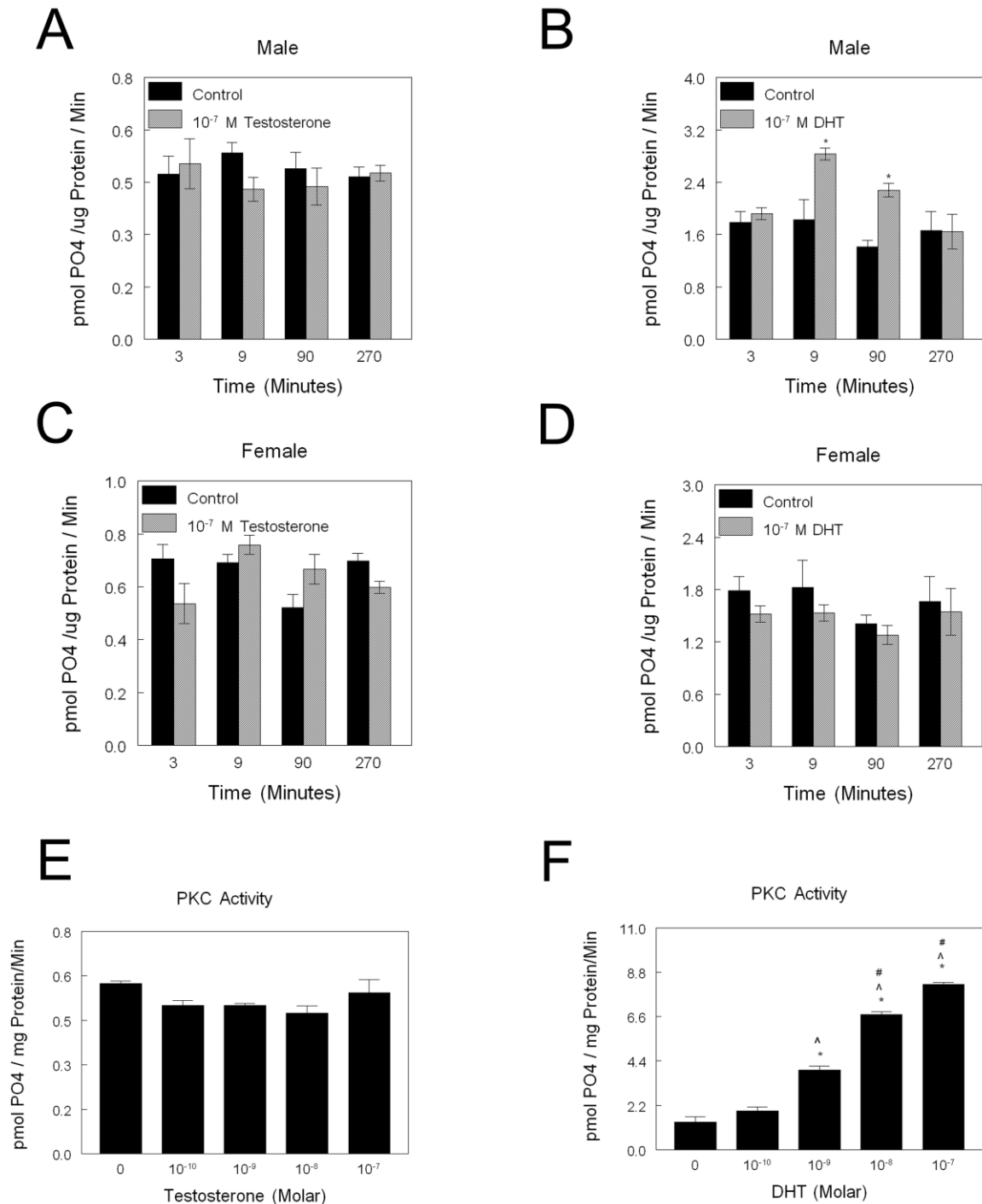
#### *Statistical Analysis*

For each experiment, each data point represents the mean $\pm$ SEM for six individual cultures (cell layers). Significance between groups was determined by Bonferroni's modification of Student's t-test using \* $P < 0.05$ . Each experiment was repeated two or more times to ensure validity of the data. The data presented are from a single representative experiment.

## Results

### *Protein Kinase C*

Testosterone had no effect on PKC in male chondrocytes at any of the times tested (Fig. 1A), whereas DHT caused a rapid increase in PKC activity at 9 minutes that was still present at 90 minutes (Figure 4.1B). Neither testosterone nor DHT affected PKC activity in female resting zone chondrocytes (Figure 4.1C, D). Testosterone failed to stimulate PKC activity in the male cells at any of the concentrations tested (Figure 4.1E). In contrast, DHT induced the PKC activity in dose dependent manner, which was significant at  $10^{-9}$  to  $10^{-7}$  M DHT (Figure 4.1F).



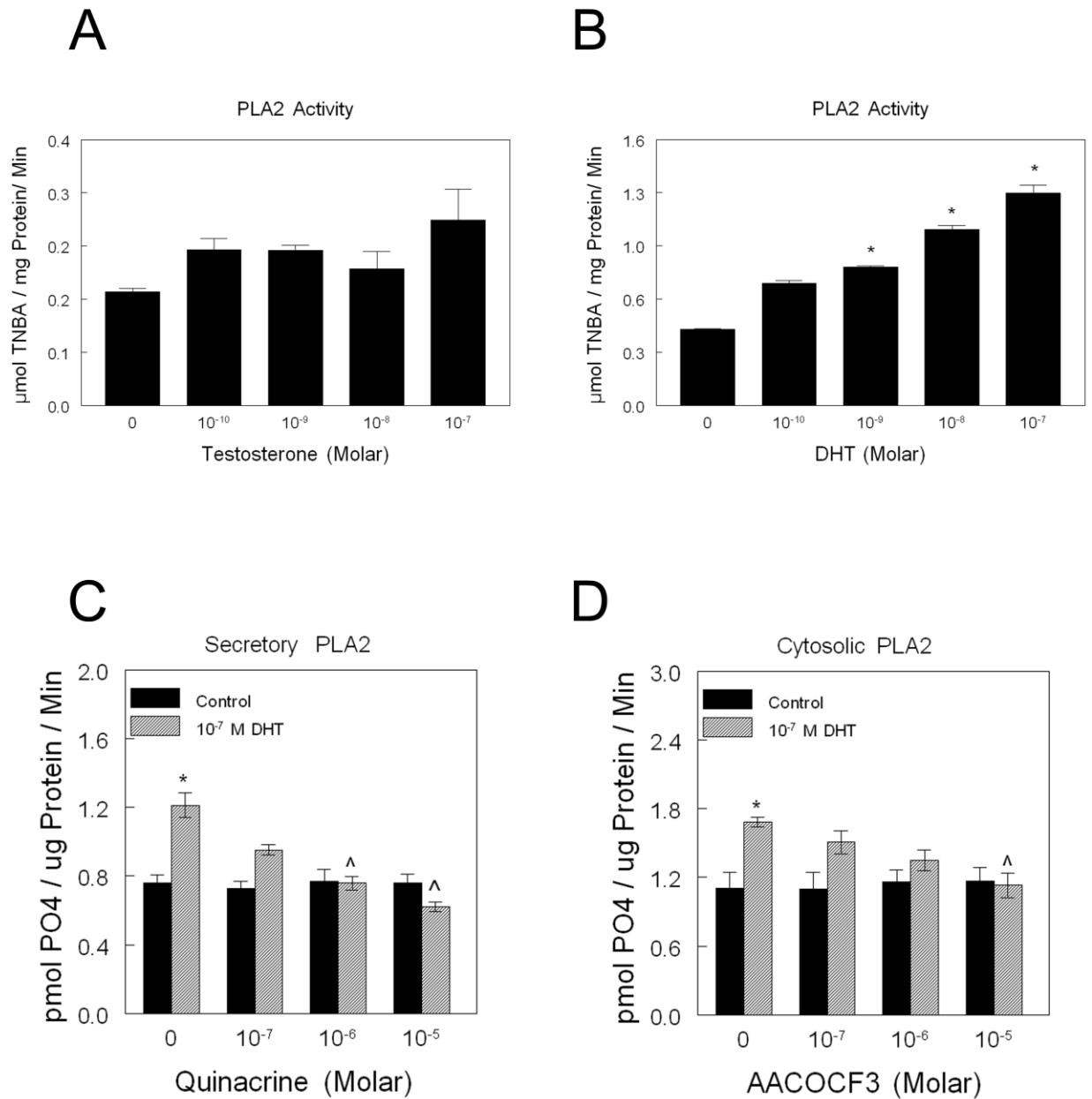
**Figure 4.1: Time- and dose-dependent effects of DHT on PKC specific activity of confluent, fourth passage rat resting zone chondrocytes.** Time-dependent response of male rat chondrocytes was evaluated at 3, 9, 90 and 270 minutes with control media or media containing  $10^{-7}$  M testosterone (4.1A) or media containing  $10^{-7}$  M DHT (4.1B). The same experimental design was used with female rat chondrocytes to evaluate the time-dependent effects of testosterone (4.1C) and DHT (4.1D). Because DHT had an effect only on male rat chondrocytes, the dose-dependent response was assessed in male

rat chondrocytes treated for 9 minutes with control media or media containing concentrations ranging from  $10^{-10}$  to  $10^{-7}$  M testosterone (4.1E) or DHT (4.1F). Data show the mean  $\pm$  SEM for six independent cultures from one representative experiment. \* $P < 0.05$ , DHT treatment vs. control, ^ $P < 0.05$   $10^{-10}$  M vs.  $10^{-9}$ — $10^{-7}$  M DHT., # $P < 0.05$   $10^{-9}$  M vs.  $10^{-8}$ — $10^{-7}$  M DHT.

### **Mechanism of DHT-dependent PKC Activation**

#### *Phospholipase A2*

Testosterone had no effect on activation of PLA2 activity in male chondrocytes (Figure 4.2A). In contrast, the effect of DHT was dose-dependent, significant from  $10^{-9}$  to  $10^{-7}$  M DHT, with a maximal effect at  $10^{-7}$  M (Figure 4.2B). Regulation of PKC activity by DHT is PLA2-dependent and both secretory and cytosolic isoforms are involved. The secretory PLA2 inhibitor quinacrine caused dose-dependent decrease in DHT-dependent PKC activity (Figure 4.2C). Similarly, the cytosolic PLA2 inhibitor AACOCF3 blocked PKC activity caused by DHT (Figure 4.2D).



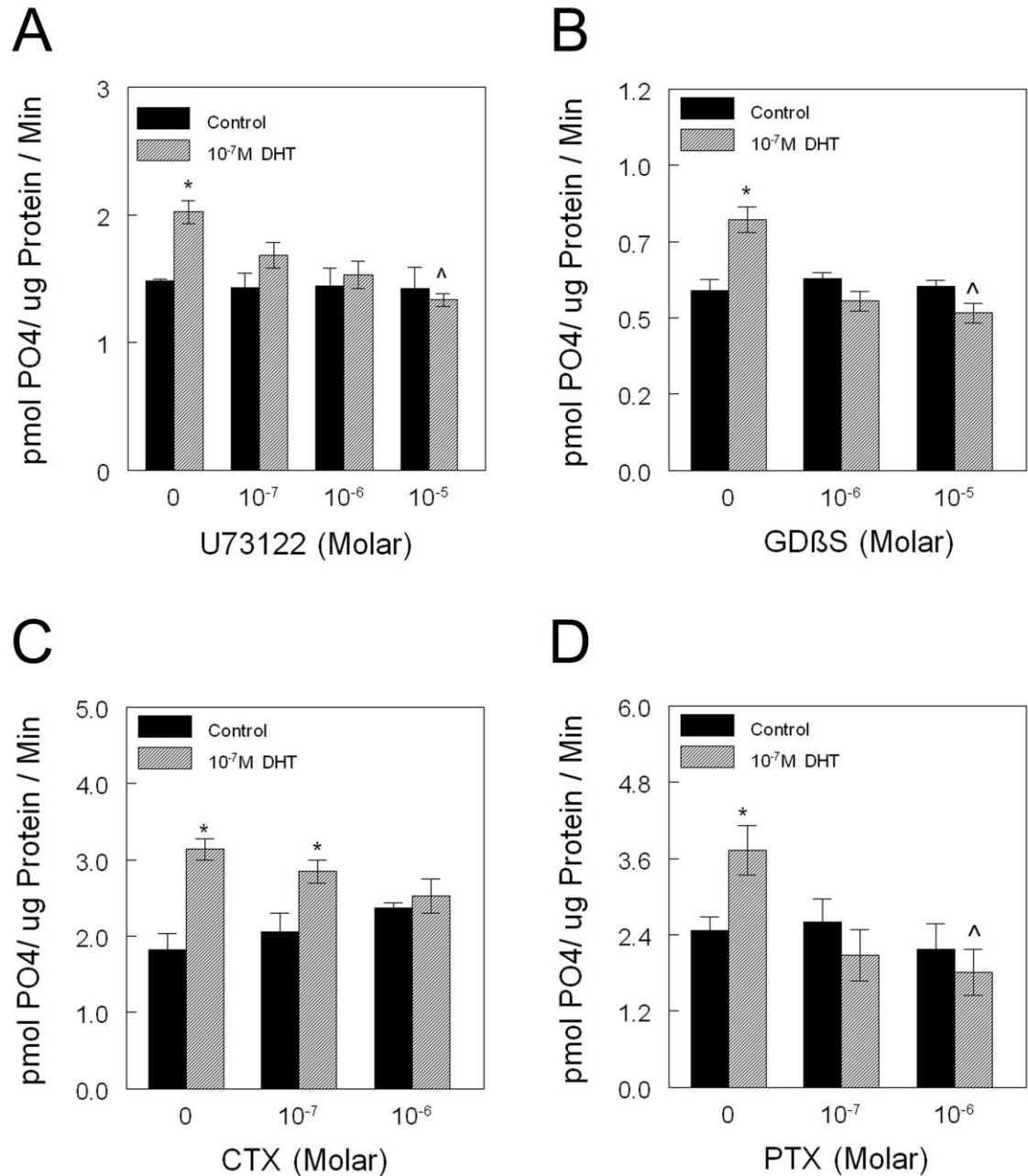
**Figure 4.2: PLA<sub>2</sub> activation and its role in DHT-dependent PKC activity in confluent, fourthpassage rat resting zone chondrocytes.** PLA<sub>2</sub> activation was measured on male rat chondrocytes treated for 9 minutes with control media or media containing concentrations ranging from 10<sup>-10</sup> to 10<sup>-7</sup> M testosterone (4.2A) or DHT (4.2B). Additionally, PKC specific activity was assayed in male rat chondrocytes treated with control media or media containing 10<sup>-7</sup> M DHT in the presence or absence of 0.1, 1 or 10 μM quinacrine (secretory-PLA<sub>2</sub> inhibitor) (4.2C) or AACOCF3 (cytosolic-PLA<sub>2</sub> inhibitor) (4.5D). Data show the mean ± SEM for six independent cultures from one representative experiment. \*P<0.05, DHT treatment vs. control; ^P<0.05 treatment vs. different inhibitors

### *Phospholipase C*

The PI-PLC inhibitor U73122 had no effect on basal PKC activity; however it inhibited PKC activity in DHT-treated male chondrocytes at  $10^{-5}$  M (Figure 4.3A).

### *G-Proteins*

G-proteins mediated the effect of DHT on PKC. The nonhydrolyzable G-protein inhibitor GDP $\beta$ S blocked DHT-stimulated PKC activity in male resting zone chondrocytes, but had no effect on the basal level activity of PKC (Figure 4.3B). Cultures treated with  $10^{-6}$  or  $10^{-5}$  GDP $\beta$ S inhibited the DHT effect on PKC activity completely.  $10^{-6}$  M cholera toxin, the activator of G $\alpha$ s, completely blocks the DHT effect on PKC activity (Figure 4.3C). Similarly,  $10^{-6}$  and  $10^{-7}$  M of the G $\alpha$ i inhibitor pertussis toxin blocked PKC activity completely (Figure 4.3D). Moreover higher concentrations of PTX ( $10^{-6}$  M) reduced PKC activity to levels below baseline.

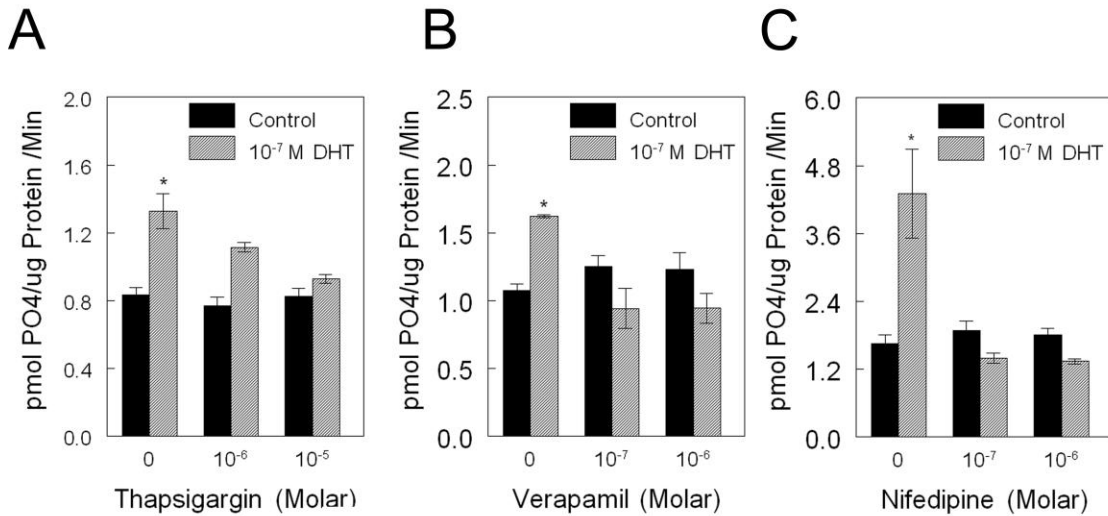


**Figure 4.3: Effect of phospholipase C inhibitor U73122 or G-protein inhibitors on DHT-dependent PKC specific activity of confluent, fourth-passage rat resting zone chondrocytes.** Male rat chondrocytes were treated for 9 minutes with control media or media containing 10<sup>-7</sup> M DHT in the presence or absence of 0.1, 1 or 10  $\mu$ M U73122 (4.3A). Alternatively, cultures were treated with DHT in the presence or absence of 1 or 10  $\mu$ M non-hydrolyzable GDP $\beta$ S (general G-protein inhibitor) (4.3B), 0.1 or 1  $\mu$ M cholera toxin (CTX, G $\alpha$ s activator) (4.3C), and 0.1 or 1  $\mu$ M pertussis toxin (PTX, G $\alpha$ i inhibitor) (4.3D). Data show the mean  $\pm$  SEM for six independent cultures from one representative experiment. \*P<0.05, DHT treatment vs. control;  $\Delta$ P<0.05 treatment vs. different inhibitors.



### *Source of Intracellular Calcium*

DHT induced PKC activity via two mechanisms: influx of extracellular  $\text{Ca}^{2+}$  via voltage gated calcium ion channels, and  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum. Thapsigargin abolished the effect of DHT on PKC activity on a dose dependent manner (Figure 4.4 A). Similarly, pre-incubation of resting zone chondrocytes from male rats with 0.1 or 1 or 10  $\mu\text{M}$  verapamil (Figure 4.4B) or nifedipine (Figure 4.4C) decreased PKC activity to baseline levels.



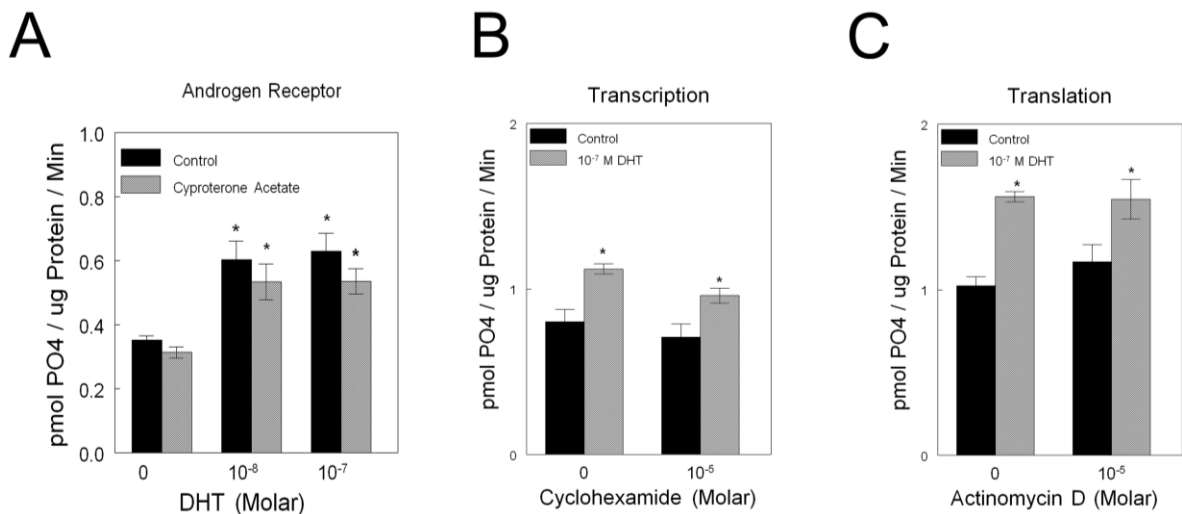
**Figure 4.4: Calcium sources involved in DHT-induced PKC activity of confluent, fourth-passage male rat resting zone chondrocytes.** Chondrocytes were treated for 9 minutes with control media or media containing 10<sup>-7</sup> M DHT in the presence or absence of 1 or 10  $\mu\text{M}$  thapsigargin (inhibitor of Ca-ATPase pumps in the endoplasmic reticulum) (4.4A). Additionally, cultures were treated with DHT in the presence or absence of 0.1 or 1  $\mu\text{M}$  verapamil ( 4.4B) and 0.1 or 1  $\mu\text{M}$  nifedipine (specific inhibitors of L-type  $\text{Ca}^{2+}$  channels on the cell membrane) (4.4C). Data show the mean  $\pm$  SEM for six independent cultures from one representative experiment. \*P<0.05, DHT treatment vs. control

### *Role of Classical Androgen Receptors*

The activation of PKC by DHT in growth plate chondrocytes isolated from male rats did not depend on classical nuclear androgen receptors (Figure 4.5A). PKC activity was not significantly changed in cultures treated with  $10^{-7}$  M CPA. Moreover, this compound did not affect basal PKC activity.

### *Role of Genomic Mechanisms in PKC Activation*

Pre-incubation of chondrocytes from male rats with the inhibitor of DNA transcription actinomycin D or the inhibitor of protein synthesis cycloheximide did not affect the ability of DHT to stimulate PKC activity (Figure 4.5 B,C).



**Figure 4.5: The role of androgen nuclear receptor and protein synthesis in DHT-induced PKC activity of confluent, fourth-passage male rat resting zone chondrocytes.** Chondrocytes were treated for 9 minutes with control media or media containing either  $10^{-8}$  or  $10^{-7}$  M DHT in the presence or absence of  $10 \mu\text{M}$  cyproterone acetate (CPA, inhibitor of classical androgen receptor) (4.5A). Additionally, cultures were treated with  $10^{-7}$  M DHT in the presence or absence of  $10 \mu\text{M}$  cycloheximide (inhibitor of protein biosynthesis) (4.5B) or  $10 \mu\text{M}$  actinomycin D (pharmacological inhibitor of DNA transcription) (4.5C). Data show the mean  $\pm$  SEM for six independent cultures from one representative experiment. \* $P < 0.05$ , DHT treatment vs. control.

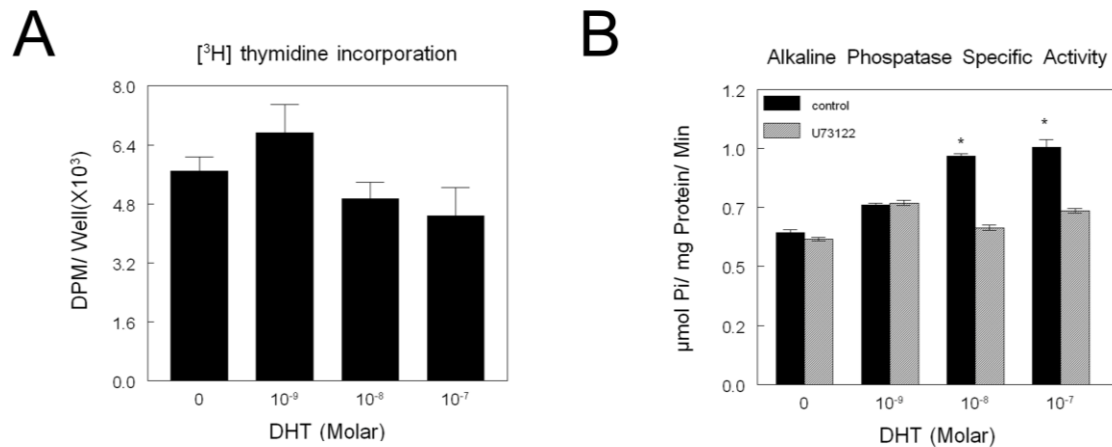
## Role of PKC in the Physiological Response to DHT

### *DNA Synthesis*

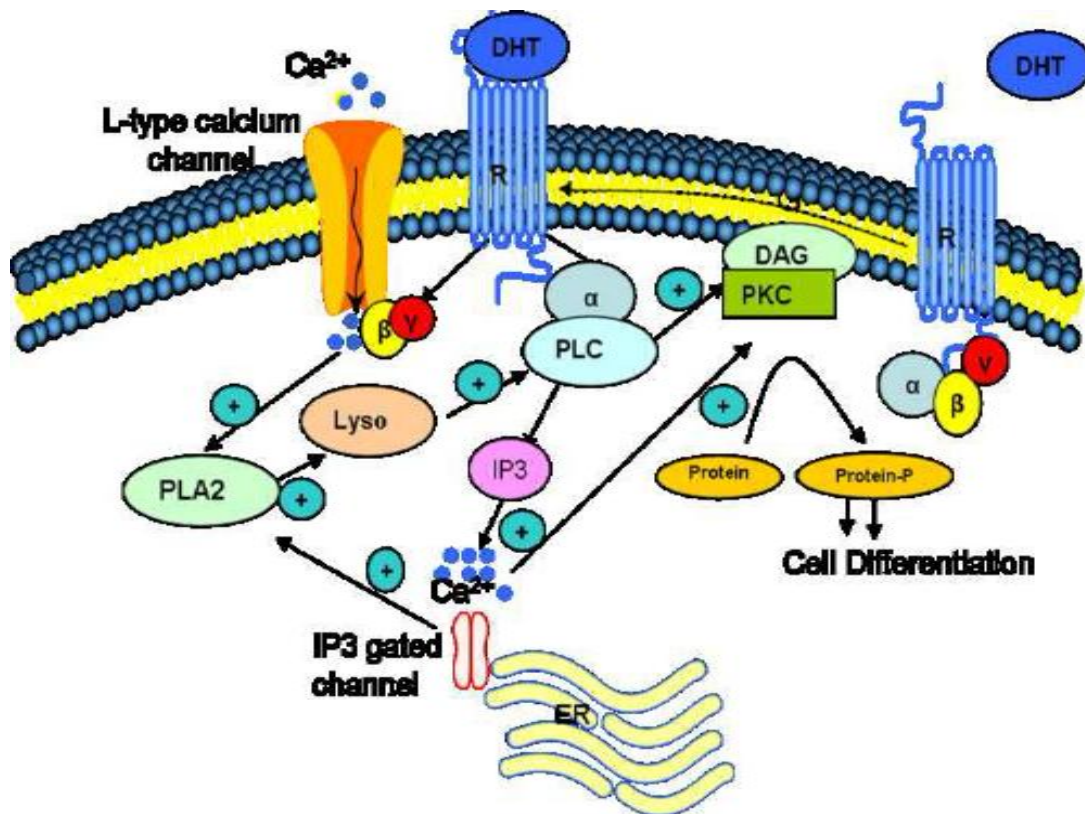
DNA synthesis was not regulated by DHT. Treatment with DHT for 9 minutes had no effect on [3H]-thymidine incorporation by resting zone chondrocytes from male rats at any concentration tested (Figure 4.6A).

### *Alkaline Phosphatase Specific Activity*

DHT regulated alkaline phosphatase in male resting zone chondrocytes. Addition of DHT for only 9 minutes caused a dose-dependent activation of alkaline phosphatase specific activity, significant at  $10^{-8}$ - $10^{-7}$  M, with the highest peak at  $10^{-7}$  M (Figure 4.6B). The regulation of alkaline phosphatase specific activity by DHT was mediated by PI-PLC since the specific inhibitor U73122 abolished the DHT effect (Figure 4.6B).



**Figure 4.6. Effect of DHT on DNA synthesis and alkaline phosphatase activity of quiescent, fourthpassage male rat resting zone chondrocytes.** Cells were treated for 9 minutes with  $10^{-9}$  to  $10^{-7}$  M DHT. To measure DNA synthesis, [3H]-thymidine was added 20 hours after DHT treatment and 4 hours prior to harvest (Fig. 6A). Alkaline phosphatase specific activity was measured in cell layer lysates 24 hours after DHT treatment in the presence or absence of  $0.1 \mu\text{M}$  PI-PLC inhibitor U73122 (Fig. 6B). Data show the mean  $\pm$  SEM for six independent cultures from one representative experiment. \* $P < 0.05$ , treatment vs. control



**Figure 4.7: Proposed mechanism of DHT signaling in male resting zone growth plate chondrocytes.** DHT exerts its effects through activation of a membrane-associated receptor (R). Stimulation of this membrane receptor initiates G $\alpha$ i (G) mediated PLA2 activation and PLC signaling pathway. A consequence of this signaling is production of inositol 1,4,5-trisphosphate (IP3) and DAG. IP3 causes an increase in the release of Ca<sup>2+</sup> from the endoplasmic reticulum (ER). Free G $\beta\gamma$  subunits produced from G-protein activation lead to an increase in Ca<sup>2+</sup> influx via L-type calcium channels. Both DAG produced as a consequence of PLC activity and increase in intracellular Ca<sup>2+</sup> activate PKC on the plasma membrane. Increase in cytosolic Ca<sup>2+</sup> as well as the stimulation of G $\alpha$ i leads to PLA2 activation and release of lysophospholipids (Lyso), which can also increase PLC activity. Once PKC is activated, it can initiate a protein phosphorylation cascade that ultimately affects cell differentiation.

## Discussion

This study demonstrates that DHT regulates growth plate chondrocytes in a sex-specific manner via rapid signaling pathways. The effects of DHT were observed only in chondrocytes from male rat growth plates and occurred within 9 minutes. Both PKC and PLA2 were activated in a dose-dependent manner and increases in activity were seen at

physiologically relevant concentrations. No effect of the testosterone metabolite was observed in the female cells. Moreover, the results show that the stimulatory effects on these two signaling pathways were specific to DHT and not testosterone as testosterone had no effect on either enzyme activity.

Neither gene transcription nor translation were required for DHT-dependent PKC activation to occur, indicating that DHT stimulated existing enzyme. We did not determine which PKC isoform was sensitive to DHT, but the assay conditions that were used are optimized to isoforms sensitive to both  $\text{Ca}^{2+}$  ions and phospholipids, suggesting a role for PKC $\alpha$  in the mechanism [196, 197].

CPA, which competes with testosterone and DHT to bind to the androgen receptor, did not inhibit the rapid responses, suggesting that DHT regulates PKC via a specific receptor that is different than the classic nuclear receptor. T-BSA which can not enter the cell, also failed to activate PKC, indicating that testosterone does not participate via a membrane form of AR and suggesting that DHT's effect is also via membrane AR. It is likely that the DHT-receptor is membrane-associated, however, as DHT stimulated PKC by mechanisms that are membrane-mediated.

The DHT-receptor might be a G-protein coupled receptor, as our results showed that G-protein is required for DHT-dependent PKC activation. There are two classes of G-proteins that couple receptors to the activation of PI-PLC: one that is PTX-sensitive and one that is PTX-insensitive [198]. Pre-incubation of chondrocytes with both PTX ( $\text{G}\alpha_i$  inhibitor) and CTX ( $\text{G}\alpha_s$  activator) completely blocked the stimulatory effect of DHT on PKC activity. In other systems, upon G-protein activation,  $\text{G}\alpha$  disassociates from  $\text{G}\beta\gamma$ , which can then activate  $\text{Ca}^{2+}$  ion channels on the plasma membrane [199]. Our

results show that DHT stimulates influx of extracellular  $\text{Ca}^{2+}$  ions. Moreover, inhibition of  $\text{Ca}^{2+}$  influx blocks the stimulatory effect of DHT on PKC.  $\text{G}\alpha$  proteins have also been shown to activate PI-PLC [200],[201] and PLC was required for DHT-dependent PKC activation, since specific inhibition of PI-PLC blocked activation of PKC by DHT. Moreover, inhibition of release of  $\text{Ca}^{2+}$  ions from the endoplasmic reticulum also reduced DHT's effect. PI-PLC catalyzes the release of IP<sub>3</sub>, which opens these  $\text{Ca}^{2+}$  ion channels and resulting DAG translocates the active PKC to the plasma membrane. Although  $\text{G}\alpha_q$  is generally associated with PI-PLC activation [202],  $\text{G}\alpha_i$  has also been shown to do so [182].

It is also possible that  $\text{G}\alpha_i$  is activating PLA<sub>2</sub>, and that is upstream of PI-PLC, as we have shown for  $1\alpha,25$ -dihydroxy vitamin D<sub>3</sub>-dependent activation of PKC in growth plate chondrocytes [203]. Our results show that DHT activated PKC via a PLA<sub>2</sub>-dependent pathway. DHT caused a rapid increase in PLA<sub>2</sub> activity and inhibition of PLA<sub>2</sub> blocked the stimulatory effect of DHT on PKC. Production of lysophospholipid [204] and release of arachidonic acid [205] are catalyzed by PLA<sub>2</sub> via phospholipid hydrolysis. Arachidonic acid can act as co-factor for PKC $\alpha$  and also provides a substrate for constitutively active cyclooxygenase-1 (Cox-1) in growth plate chondrocytes, which leads to prostaglandin production. In addition, lysophospholipid can activate PLC [203], and can then initiate the downstream PLC-PKC pathway (Fig. 7).

Our results demonstrate that DHT regulates proliferation and differentiation of growth plate chondrocytes by multiple mechanisms. The fact that U73122 blocked the stimulatory effect of DHT on alkaline phosphatase activity suggests that rapid membrane response via the PLC-PKC pathway plays an important role in

differentiation of male chondrocytes. In contrast, DNA synthesis is not regulated in this manner. We previously showed that testosterone and DHT can regulate proliferation [33]; however, in the present study, a 9 minute pulse of DHT had no effect on [ $^3\text{H}$ ]-thymidine incorporation, indicating that the proliferative effects of testosterone and/or DHT are likely mediated by the classic androgen receptor.

Rat growth plate chondrocytes possess  $5\alpha$ -reductase and convert testosterone to DHT [33, 172], and regulation of these cells by testosterone is dependent on the production and release of DHT [172]. Here we show that DHT but not testosterone elicits the rapid membrane response. Taken together, this suggests that the membrane-associated effects of androgens are mediated by paracrine actions of DHT.

In summary, DHT regulates male resting zone chondrocytes through rapid activation of a membrane-associated receptor. This results in PLA2 and PLC-mediated activation of PKC and increase in chondrocyte differentiation. These findings indicate the importance of the membrane effects as mediators of DHT action in chondrocytes and suggest the existence of a specific membrane receptor for DHT in chondrocytes.

## **CHAPTER 5**

### **INVOLVEMENT OF RECEPTORS FOR ANDROGEN AND ESTROGEN IN THE SEX-SPECIFIC RESPONSE OF GROWTH PLATE CHONDROCYTES TO DIHYDROTESTOSTERONE**

#### **Introduction**

Androgen receptors (ARs) and estrogen receptors (ERs) are found together in many tissues, and their interactions may be responsible for a variety of physiological effects including normal development as well as pathology [206]. However, their interaction at the molecular level has been largely undefined [207]. Estrogens and androgens interact in prostate cells to modulate androgen actions [208]. One possibility is that estrogens and androgens induce assembly of a ternary complex. In LNCaP prostate adenocarcinoma cells, this complex is hypothesized to consist of the AR, ER $\beta$  and Src [209]. Its formation can be triggered by 17 $\beta$ -estradiol or by the synthetic androgen methyltrienolone (R1881), which activates Src-dependent S-phase entry and cell proliferation. The AR antagonist Casodex inhibits this effect, further supporting the hypothesis of an ER/AR complex.

Two natural potent androgens, testosterone and its metabolite dihydrotestosterone (DHT), are found in humans and mammals [10]. Both androgens interact with the AR to regulate androgen-target gene expression. DHT, which is converted from testosterone by 5 $\alpha$ -reductase isozymes, is the major intracellular androgen and the major mediator of androgen actions in the prostate [11]. Like other steroid hormones, testosterone and DHT bind to intracellular ARs, inducing receptor dimerization. This facilitates the ability of ARs to bind to DNA and recruit coregulators to promote or suppress the expression of target genes [12, 13]. This mechanism of steroid action is known as the classical or



genomic pathway and because it involves gene transcription and mRNA translation, its effects may take several hours to days to be seen [14].

In addition to their genomic actions, it is now understood that steroid hormones can also exert actions that are insensitive to inhibitors of transcription and translation and occur within seconds to minutes after administration of the hormone [15, 16]. This mechanism of steroid action is referred to as non-genomic or non-classical and may result in changes in downstream gene expression via membrane-associated signaling pathways [17, 18]. Several studies show that these rapid effects of androgens are mediated by cellular signaling pathways involving surface membrane receptors and second messengers [19, 20]. In cultured cardiac myocytes, testosterone has been found to induce a rapid increase in intracellular  $\text{Ca}^{2+}$  through activation of a plasma membrane AR associated with the pertussis toxin (PTX)-sensitive G protein-coupled phospholipase C (PLC), inositol trisphosphate (IP3) signaling pathway [21, 22]. Similarly DHT induces protein kinase C (PKC) activity via a PLC dependent pathway [23].

Sex steroids can elicit sex-specific effects in their target cells via membrane associated mechanisms. The ability of testosterone or testosterone coupled to bovine serum albumin (T-BSA), which cannot cross the membrane, to increase  $\text{Ca}^{2+}$  in primary osteoblasts is found only in cells from male rats but not from female rats [24]. Importantly, the rapid effect of androgens on PKC in costochondral cartilage cells is specific to DHT and is found only in chondrocytes from male animals. Testosterone does not induce this response in cells from rats of either sex. Estrogens also regulate cells by rapid membrane associated signaling pathways in addition to classical ER-dependent mechanisms [25] and some of the membrane-associated effects are sex-specific [24].

Rat growth plate chondrocytes possess ARs, ER $\alpha$  and ER $\beta$  [74, 75, 210] and recent studies show that ER $\alpha$  is present in the plasma membrane in different molecular forms: the traditional 68kDa nuclear form (ER $\alpha$ 68), a 46kDa form (ER $\alpha$ 46) and a 36kDa form (ER $\alpha$ 36) [45]. ER $\alpha$ 36 is responsible for some of the rapid actions of estrogen that are mediated by membrane associated signaling pathways [211]. An AR splice variant was recently identified in the plasma membrane of prostate cancer cells [212]. It lacks a DNA binding domain and therefore, may not function as a transcription factor on its own but it may contribute to the membrane associated response to DHT. Because ARs and ERs do not possess an intrinsic transmembrane domain [76], it is likely that they interact with specific membrane protein(s) in order to associate with the plasma membrane [77, 78]. Caveolin-1 has been implicated as has palmitoylation as a mechanism for locating ERs at the plasma membrane [72, 73]34], suggesting that similar mechanisms are involved in AR translocation.

In long bones, endochondral bone formation occurs at the growth plate, a region of developing cartilage located between the epiphysis and the metaphysis [171]. The process of endochondral ossification is regulated in part by sex steroid hormones [27]. Androgens stimulate endochondral bone growth and elongation [28, 213], while estrogen is known to suppress longitudinal bone growth and accelerate growth plate closure [29, 33, 34, 75, 172-175]. Studies using rat costochondral growth plate chondrocytes as a model show that the effects of 17 $\beta$ -estradiol (E<sub>2</sub>) on apoptosis are found in both male and female cells and the same mechanism is involved [176]. In contrast, E<sub>2</sub> causes rapid activation of PKC in female cells but not in male cells. DHT also has direct effects on growth plate chondrocytes, increasing matrix synthesis including sulfated

glycosaminoglycan production [214], and enhancing cell maturation by increasing alkaline phosphatase enzymatic activity [23]. Moreover, these effects of DHT are sex-specific and depend on rapid PKC dependent signaling [23]

The goal of this study was to investigate the role of the ARs and ERs in the rapid membrane-associated actions of DHT, using sex-specific DHT-dependent stimulation of PKC in male rat costochondral cartilage cells as a model. Differences in response to DHT and its enantiomer *ent*-DHT were used to determine if DHT acts via direct receptor binding or indirect membrane perturbation effects [215]. We used selective agonists and antagonists of the AR, as well as specific blocking antibodies for ER $\alpha$  and ER $\beta$  to determine if one or both receptors mediate the response. We assessed the plasma membrane location of the receptors by determining whether they co-localize with caveolin-1 (Cav-1) and if intact caveolae are required for the response. We determined if palmitoylation or myristylation of the receptor(s) is involved in targeting the receptor(s) to the plasma membrane. Finally, we assessed the role of the AR in regulating growth plate chondrocyte maturation by determining the effect of DHT-dependent signaling on [ $^{35}$ S]-sulfate incorporation.

## **Materials and Methods**

### ***Reagents***

5 $\alpha$ -Dihydrotestosterone (DHT) and methyl- $\beta$ -cyclodextrin ( $\beta$ -CD) were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-ER $\alpha$  monoclonal antibody (ab16460), anti-ER $\beta$  monoclonal antibody (ab16813), non-specific IgG (ab91353), and non-specific IgM (ab91545) were purchased from Abcam (San Francisco, CA). A polyclonal antibody to Cav-1 was purchased from Santa Cruz Biotechnology (sc-894, Santa Cruz, CA) The

AR inhibitor bicalutamide (Casodex) [216] was obtained from Tocris Cookson, Inc. (Ellisville, MO). Tunicamycin, an inhibitor of palmitoyltransferase [113], was purchased from EMD Chemicals, Inc. (San Diego, CA). 2-Hydroxymyristic acid (HMA), a myristate antagonist [113], was obtained from Cayman Chemical (Ann Arbor, MI). A PKC assay kit was obtained from Amersham Bioscience (Piscataway, NJ) and Dulbecco's modified Eagle medium (DMEM) was obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay kit [87] obtained from Pierce Chemical Co. (Rockford, IL). [<sup>32</sup>P]-ATP; the synthetic androgen, methyltrienolone R1881; and [<sup>35</sup>S] were obtained from Perkin Elmer (Melville, NY).

### ***Chondrocyte Cultures***

The culture system used in this study has been described in detail previously [147]. Chondrocytes were isolated from the resting zone cartilage of the costochondral junction of 150g male Sprague-Dawley rats. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml sodium ascorbate in an incubator at 5% CO<sub>2</sub> and 100% humidity at 37°C. Fourth passage cells were used for all experiments based on previous studies showing that these cells preserve their chondrocyte phenotype as well as their differential responsiveness to vitamin D metabolites at this passage [89].

### ***Regulation of Protein Kinase C Activity***

Initial experiments determined the receptor(s) involved in regulation of PKC activity by DHT. Confluent cultures were treated for 9 minutes either with vehicle alone (0.5 ml of ethanol at highest concentration used in experimental cultures) or experimental

media (DMEM + 10% FBS + various concentrations of *ent*-DHT [54] or R1881). Cultures were treated with (DMEM + 10% FBS +  $10^{-7}$ M DHT) as the positive control [23]. The contribution of ARs to any change in PKC activity was determined by pre-incubating cells for 30 minutes with  $10^{-5}$ M Casodex followed by 9 minutes with  $10^{-7}$ M DHT.

To determine the involvement of ERs in this pathway, cells were pre-incubated for 30 minutes with 1:500 v/v dilution of non-specific antibodies IgG and IgM or with specific monoclonal antibodies to ER $\alpha$  and ER $\beta$ , respectively. At the end of the pre-incubation, the cells were treated for 9 minutes with vehicle alone or with  $10^{-7}$ M DHT. Although these monoclonal antibodies were generated to intracellular ERs, they have been used successfully to label protein in the plasma membrane [217]. To assess the role of palmitoylation or myristylation of AR in regulating PKC activity, cells were pre-incubated for 2 hours with 30 $\mu$ M tunicamycin [218] or with 0.5mM 2-hydroxymyristic acid (HMA) [218], and then with  $10^{-7}$  M DHT for 9 minutes.

### ***Requirement for Caveolae***

To determine if the DHT-dependent increase in PKC requires caveolae, cultures were pre-incubated for 30 minutes with 5mM  $\beta$ -CD in order to disrupt the caveolae by depleting them of cholesterol [72, 140]. Cells were then treated for 9 minutes with  $10^{-7}$  M DHT as described previously [140]. In order to verify that ARs are present in caveolae, we used a detergent-free method to isolate and fractionate plasma membranes. Chondrocytes were treated with either  $10^{-7}$ M DHT or vehicle for 9 minutes. The cells layers were washed two times with phosphate buffered saline and the cells were harvested by scraping using isolation buffer (0.25M sucrose, 1mM EDTA, 20mM tricine,

pH 7.8). The cells were homogenized using a tissue grinder and the homogenate was centrifuged at 20,000 x g for 10 minutes. The pellet, including nuclei, mitochondria, and endoplasmic reticulum was discarded. The supernatant was collected and placed on top of isolation buffer containing 30% Percoll (GE Healthcare, Piscataway, NJ). After centrifuging at 84,000 x g for 30min, the plasma membrane fraction appeared as a visible band in the middle of the centrifuge tube. This fraction was layered over a 10–20% OptiPrep gradient (Sigma–Aldrich), and the gradient was centrifuged at 52,000 x g for another 4 hours. Plasma membrane sub-fractions were collected from the top to the bottom of the tube, which resulted in isolation of 15 fractions. Caveolae were observed as an opaque band, which was collected in fraction 3, based on enrichment in caveolin-1 by western blot analysis.

Whole cell lysates, plasma membranes and the caveolae fraction 3 were separated on 4-20% gradient acrylamide gels. Blots of the gels were probed with the rabbit monoclonal antibody against AR (ab16460) and rabbit polyclonal antibody against Cav-1. Immunoreactive bands were visualized using goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). The Super Signal West Pico Chemiluminescent System (Thermo Fisher Scientific) was used to treat the membrane, which was then imaged with the VersaDoc imaging system (Bio-Rad).

### ***Role of ARs in Chondrocyte Response to DHT***

Previous studies show that DHT stimulates [<sup>35</sup>S]-sulfate incorporation by rat costochondral cartilage cells [174]. To determine if this effect is mediated by the AR via membrane associated signaling pathways, we first determined if transient exposure to DHT could trigger the response. 10<sup>-9</sup>- 10<sup>-7</sup>M DHT was added to confluent cultures for 9

minutes. The media were removed and fresh media were added for 24 hours. Four hours prior to harvest, 50  $\mu$ l DMEM containing 18  $\mu$ Ci/ml [ $^{35}$ S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [ $^{35}$ S]-sulfate incorporation determined as a function of protein in the cell layers [87].

To determine if the effect of DHT on [ $^{35}$ S]-sulfate incorporation was mediated by the AR, confluent cultures were pre-incubated with the AR inhibitor  $10^{-5}$  M Casodex for 30 minutes, followed by  $10^{-8}$ -  $10^{-7}$  M DHT for 9 minutes. The same strategy was used to assess the involvement of PLC. Cells were pre-incubated for 30 minutes with  $10^{-5}$  M U73122 to inhibit phosphatidylinositol-dependent PLC, followed by  $10^{-8}$ -  $10^{-7}$  M DHT for 9 minutes. Experimental media were removed and fresh media were added for 24 hours.

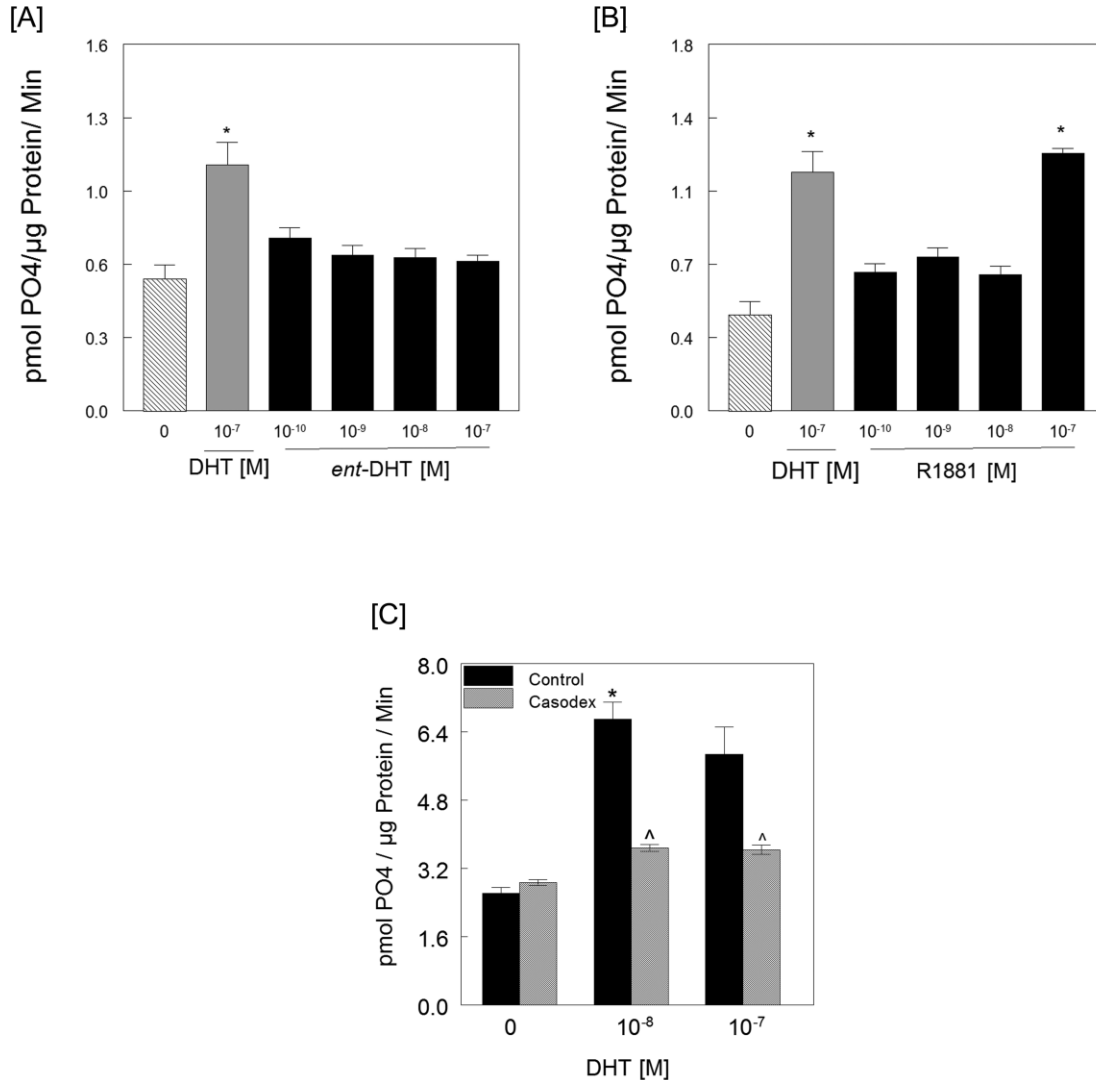
### ***Statistical Management of the Data***

For each experiment, each value represents the mean  $\pm$  SEM of the cell layers of six independent cultures. Statistical significance was determined by ANOVA, followed by Bonferroni's modification of Student's t-test.  $P < 0.05$  was considered significant. Each experiment was repeated two or more times to ensure the validity of the results. The data presented are from a single representative experiment.

### **Results**

The effect of DHT on PKC is receptor-mediated. Only DHT, but not *ent*-DHT, caused a concentration dependent increase in PKC specific activity in male cells (Figure 5.1A). The AR is involved in the mechanism. The selective AR agonist R1881 activated

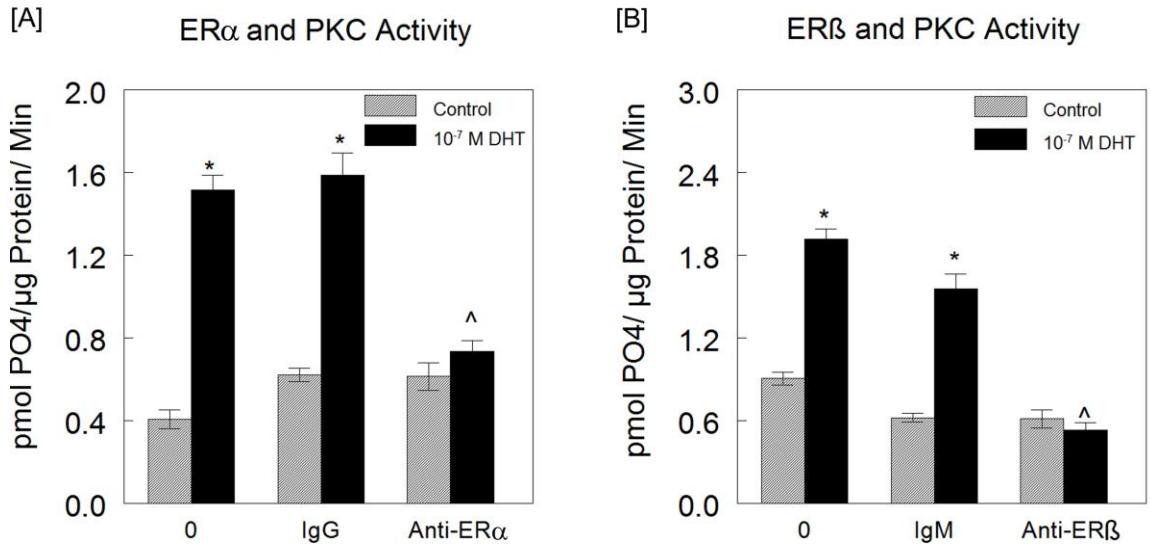
PKC (Figure 5.1B), whereas the AR specific antagonist Casodex completely abolished the effect of DHT (Figure 4.1C).



**Figure 5.1: Dose-dependent effect of *ent*-DHT, R1881 and role of AR in PKC specific activity in male resting zone chondrocytes.** Dose dependent effects were determined by treating male cells for 9 minutes with 10<sup>-9</sup> to 10<sup>-7</sup> M *ent*-DHT(A) or 10<sup>-9</sup> to 10<sup>-7</sup> M R1881 (B), or with or 10<sup>-7</sup> M DHT as a positive control. Role of AR was tested by treating the cells for 9 minutes with 10<sup>-7</sup> M DHT ± 10<sup>-5</sup> M casodex (C). PKC specific activity was measured in cell layer lysates. Values are the mean±SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control at each time point.



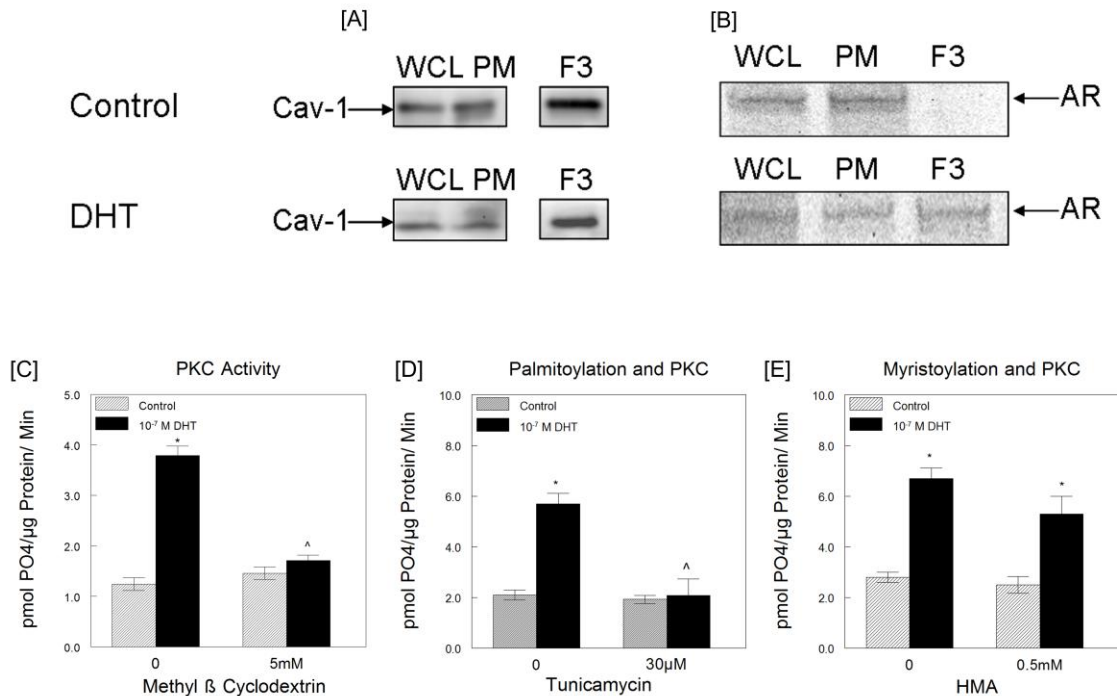
ERs also play a role in mediating the effect of DHT on PKC. Antibodies to either ER $\alpha$  (Figure 5.2A) or ER $\beta$  (Figure 5.2B) completely blocked DHT-dependent activation of PKC, whereas IgM and IgG had no effect.



**Figure 5.2: Involvement of ER $\alpha$  and ER $\beta$  in DHT-mediated PKC activity.** Confluent male resting zone chondrocytes were pre-incubated for 30 minutes with anti-ER $\alpha$  antibody (A) or anti-ER $\beta$  antibody (B). Cells were then treated with 10<sup>-7</sup> M DHT for 9 minutes. PKC specific activity was measured in cell layer lysates. Values are the mean $\pm$ SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control,  $\Delta$ P<0.05, DHT+ anti-ER $\alpha$  vs. DHT alone or DHT + anti-ER $\beta$  vs. DHT alone for each experiment.

The mechanism by which the AR mediates DHT's effects involves caveolae. Western blots of whole cell lysates, plasma membranes and plasma membrane fraction 3 confirmed the presence of caveolin-1 (Figure 5.3A). After DHT treatment for 9 minutes, the level of AR in the plasma membrane was reduced and AR was concentrated instead in fraction 3 (Figure 5.3B), indicating translocation to caveolae is required. Treatment of rat resting zone chondrocytes with 5mM  $\beta$ -CD abrogated the DHT-dependent increase in PKC (Figure 5.3C), supporting the hypothesis that lipid rafts and/or caveolae play a role. Palmitoylation was required for localization of AR to the membrane. Tunicamycin

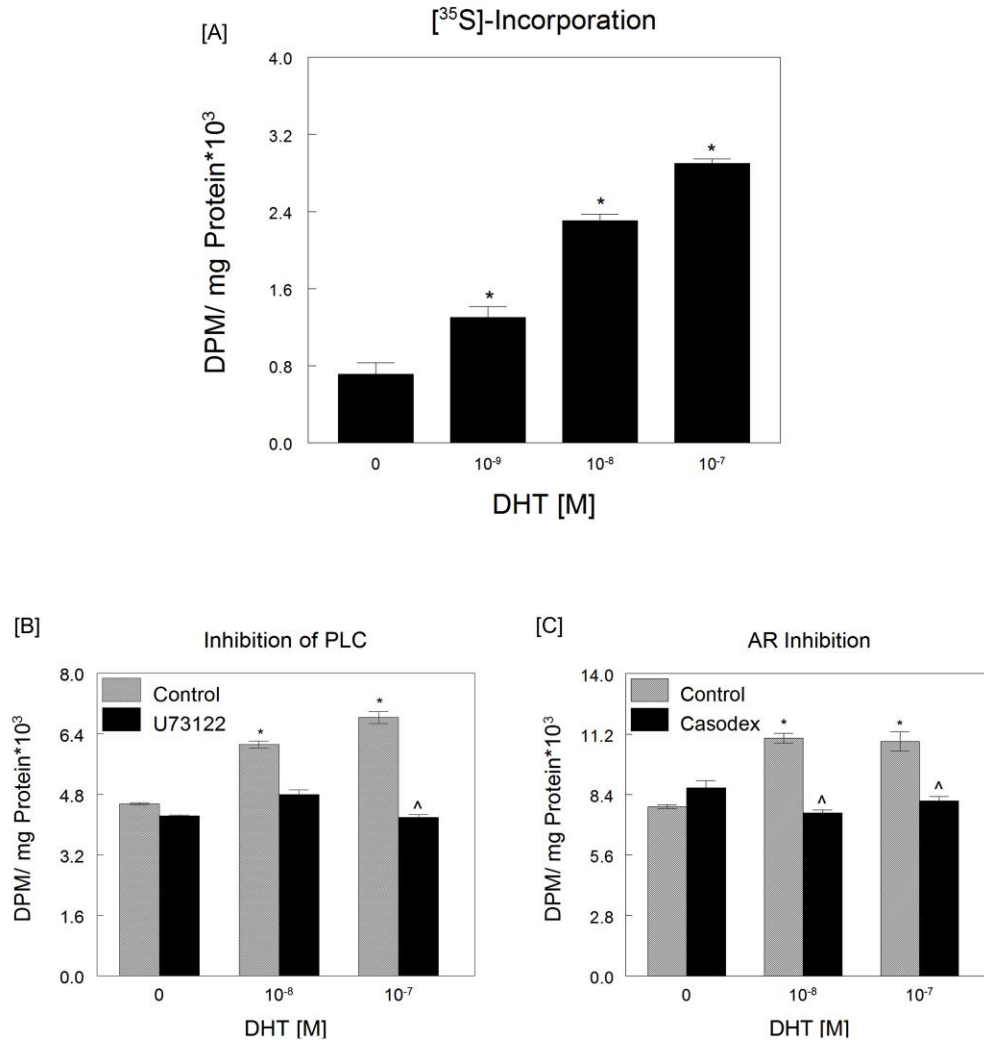
(30 $\mu$ M) was able to completely abolish the effect of DHT on PKC activity (Figure 5.3D). However, localization of AR in caveolae did not involve myristylation, as HMA had no effect (Figure 5.3E).



**Figure 5.3. Subcellular localization of AR in male resting zone chondrocytes.** Male resting chondrocytes were treated for 9 minutes  $\pm$  10<sup>-7</sup> M DHT. Whole cell lysates (WCL), plasma membranes (PM), and plasma membrane fraction 3 (caveolae) were isolated and subjected to western blot (A,B). Role of caveolae structure and AR palmitoylation on DHT-mediated PKC activity in male resting zone chondrocytes. Cultures were pre-incubated for 30 minutes with 5mM methyl- $\beta$ -cyclodextrin ( $\beta$ -CD); then cells were treated for 9 minutes with 10<sup>-7</sup> M DHT(C). Cultures were pre-incubated for 2 hours with 30 $\mu$ M tunicamycin (D), or 0.5mM 2-hydroxymyristic acid (E). Then cells were treated for 9 minutes with 10<sup>-7</sup> M DHT. PKC specific activity was measured in cell layer lysates. Values are the mean  $\pm$  SEM for six independent cultures for each variable. \*P<0.05, treatment vs. control, ^P<0.05, DHT+  $\beta$ -CD or DHT+ tunicamycin vs. DHT alone for each specific experiment.

Chondrocytes treated with DHT exhibited increased [<sup>35</sup>S]-sulfate incorporation in a dose dependent manner (Figure 5.4A). This was mediated by the AR, as the AR-selective antagonist Casodex blocked the effect (Figure 5.4B). The effect of DHT on

[<sup>35</sup>S]-sulfate incorporation required PLC since inhibition of PI-PLC with U73122 blocked the DHT-dependent increase (Figure 5.4C).



**Figure 5.4: DHT increases maturation in resting zone chondrocytes.** Chondrocyte maturation was determined by measuring [<sup>35</sup>S]-incorporation. Male rat resting zone chondrocytes were treated for 9 minutes with 10<sup>-9</sup> - 10<sup>-7</sup>M DHT alone (A) or cells were pre-incubated for 30 minutes with 10<sup>-5</sup> M U73122 to inhibit PLC (B) or for 30 minutes with 10<sup>-5</sup> M Casodex (C). Cells were then treated for 9 minutes with 10<sup>-7</sup> M DHT. Media were removed and fresh media were added for 24 hours. Four hours prior to the harvest, cells were labeled with [<sup>35</sup>S]-sulfate. At harvest, the conditioned media were removed, the cell layers (cells and matrix) were collected, and the amount of [<sup>35</sup>S]-sulfate incorporated determined as a function of protein in the cell layer. Data are the mean ± SEM for six independent cultures from one representative experiment. \*P<0.05, <sup>Δ</sup>P<0.05.

treatment vs. control.  $^{\wedge}P<0.05$ , DHT+ U73122 vs. DHT alone or DHT + Casodex vs. DHT alone for each experiment.

## Discussion

Our previous studies demonstrated that DHT can rapidly activate PKC via a pathway that involves G-protein-dependent PI-PLC only in male chondrocytes. However, the actual receptors that mediate DHT-stimulated PKC were not determined. That the effect of DHT is receptor mediated was demonstrated in the present study by the fact that only DHT but not *ent*-DHT increased PKC activity. Because enantiomers are structural mirror images with identical physicochemical properties and receptors have well-defined and structurally discriminate binding pockets, they can generally identify specific ligands of different absolute configurations. In contrast, membrane lipids present a dynamic environment that does not maintain structurally well-defined binding sites for steroids [102]. Thus, the difference in the effect of each enantiomer (DHT and *ent*-DHT) on the cell response implies that the binding affinity of DHT to the specific receptor is more effective than its enantiomer (*ent*-DHT).

Our results demonstrate that both ER $\alpha$  and ER $\beta$  are involved in mediating the rapid membrane-associated effects of DHT on the PKC signaling pathway. Antibodies specific for ER $\alpha$  and ER $\beta$  blocked the stimulatory effects of DHT on PKC activity. ER $\alpha$  [105] and ER $\beta$  [106], as well as truncated forms of both receptors are present in the plasma membrane [107, 108] and caveolae [109, 110], implying that membrane forms of these receptors may play a role in mediating rapid responses to the hormone via caveolae-dependent mechanisms. This is supported by the observation that destruction of caveolae with  $\beta$ -CD blocked the stimulatory effect of DHT on PKC.

Further evidence for the role of caveolae in mediating the effects of DHT on PKC, is translocation of the AR to this specialized membrane domain in response to the hormones. ARs do not display an intrinsic transmembrane domain [219]; thus an interaction of ARs with specific membrane proteins has been proposed to explain their membrane localization [219, 220]. The AR has been shown to interact with caveolin-1 in other systems [221]. We did not address this specifically but our finding supports this hypothesis. Caveolin-1 exists primarily in fraction 3 of the plasma membrane and AR was present in fraction 3 only after treatment with DHT.

We found that posttranslational palmitoylation is required for AR membrane localization and for DHT-induced recruitment of ARs to caveolae. It is possible that by palmitoylation, ARs are dynamically recruited from the cytoplasmic pool to the plasma membrane and specifically to caveolae, which contain other signaling molecules including G-protein  $\alpha$ -subunits, kinases, and caveolins [114]. Palmitoylation seems to be a common regulatory mechanism in the formation of membrane-associated signaling complexes. The interaction between palmitoylated ARs and other caveolae proteins may act as a signal for the directional recruitment and restructuring of the receptor. .

This study demonstrates that DHT signaling via the AR plays a role in chondrocyte maturation by increasing [ $^{35}\text{S}$ ]-sulfate incorporation, suggesting an increase in the synthesis of a sulfated proteoglycan extracellular matrix. Alkaline phosphatase specific activity also increased [23], supporting the hypothesis that the chondrocytes produce a mature matrix containing alkaline phosphatase-rich extracellular matrix vesicles [222] in response to DHT. Importantly, our data demonstrate that this effect of

DHT is mediated by AR-dependent signaling. It was blocked by AR antagonist, Casodex and by the PLC inhibitor U73122.

## **CHAPTER 6**

## CONCLUSIONS AND FUTURE PERSPECTIVES

Our studies show that female growth plate resting zone chondrocytes respond to estrogen with an increase in both PKC and PLA<sub>2</sub> activities via ER $\alpha$  and ER $\beta$ . That the effect of E<sub>2</sub> is specific and receptor mediated was demonstrated by the fact that only E<sub>2</sub> but not *ent*-E<sub>2</sub> had an effect on PKC activation. Because enantiomers are structural mirror images with identical physicochemical properties and receptors have well-defined and structurally discriminate binding pockets, they can generally identify specific ligands of different absolute configurations. In contrast, membrane lipids present a dynamic environment that does not maintain structurally well-defined binding sites for steroids [102]. Thus, the difference in the effect of each enantiomer (E<sub>2</sub> and *ent*-E<sub>2</sub>) on the cell response implies that the binding affinity of E<sub>2</sub> to the specific receptor is more effective than its enantiomer (*ent*-E<sub>2</sub>). This supports our previous work showing that the stimulatory effect of E<sub>2</sub> on PKC signaling is stereospecific [61, 67].

Our results demonstrate that both ER $\alpha$  and ER $\beta$  are involved in mediating the rapid membrane-associated effects of E<sub>2</sub> on PKC and PLA<sub>2</sub> signaling pathways. Antibodies specific for ER $\alpha$  and ER $\beta$  blocked the stimulatory effects of E<sub>2</sub> on both enzymes. In addition, specific agonists for ER $\beta$  and ER $\alpha$  stimulated PKC with dose-response and time course comparable to that of E<sub>2</sub>. R,R-THC, which is an agonist for ER $\alpha$  and an antagonist for ER $\beta$  had no effect on PKC, further supporting the requirement for both receptors.

The observation that E<sub>2</sub> treatment resulted in co-immunoprecipitation of ER $\alpha$ /ER $\beta$ , suggests that a complex is formed. We did not test whether such a complex is

required for membrane associated signal transduction to occur, but the fact that antibody-blocking of selective agonists for each receptor blocked the effect of E<sub>2</sub> is strong presumptive evidence that this is the case. These results support previous studies showed that ER dimerization promotes selective G-protein activation and the loss of the dimer fails to provide the structural contact points for G-proteins to bind in an active conformation, thus impairing signaling to adenylate cyclase [103]. ERs have also been shown to form a complex with androgen receptor in response to treatment with E<sub>2</sub> or dihydrotestosterone [104].

ER $\alpha$  [105] and ER $\beta$  [106], as well as truncated forms of both receptors are present in the plasma membrane [107, 108] and caveolae [109, 110], implying that membrane forms of these receptors may play a role in mediating rapid responses to the hormone. We did not address this hypothesis directly in the present study since the immunoprecipitation studies were performed using cell lysates and not isolated plasma membranes. However, our results do show that palmitoylation is required for rapid activation of PKC by E<sub>2</sub>, indicating that receptor recruitment to the membrane is involved [111, 112]. In contrast, inhibition of myristylation had no effect on E<sub>2</sub>-dependent PKC activation, indicating that the ERs responsible for mediating the hormone's effect were not integral to the plasma membrane via this mechanism [113].

ERs do not display an intrinsic transmembrane domain [76]; thus an interaction of ERs with specific membrane protein has been proposed to explain their membrane localization [77, 78]. The interaction between ERs caveolin-1 and palmitoylation of ERs are the major determinants for the residence of ERs at the plasma membrane [79-81]. In this study, we found that posttranslational palmitoylation is required for ER membrane



localization and for E<sub>2</sub>-induced recruitment of ERs to the plasma membrane. It is possible that by palmitoylation, ERs are dynamically recruited from the cytoplasmic pool to the plasma membrane and most likely to the caveolae, which contains other signaling molecules including G-protein  $\alpha$ -subunits, kinases, and caveolins [114]. The interaction between palmitoylated ERs and other caveolae proteins may act as a signal for the directional recruitment and restructuring of the receptor. Consequently, ERs were able to mediate further stimulation of PKC activity at 90 minutes. Thus, palmitoylation seems to be a common regulatory mechanism in the formation of membrane-associated signaling complexes [115]. This study provides evidence that the sex-specific difference in E<sub>2</sub>-dependent membrane-associated signaling male and female growth plate chondrocytes is due to differential regulation of ER isoforms in the plasma membrane and specifically, in the caveolae. Whereas plasma membranes and caveolae isolated from female cells possess ER $\alpha$ 68, ER $\alpha$ 46 and ER $\alpha$ 36 as well as ER $\beta$ 59, plasma membranes from male cells lack ER $\alpha$ 36. Moreover, caveolae from male cells lack both ER $\alpha$ 36 and ER $\beta$ 59. Caveolae are critical to the ability of E<sub>2</sub> to activate rapid PKC signaling in the female cells, as evidence by the loss of an E<sub>2</sub> effect when caveolae were disrupted by treatment with  $\beta$ -cyclodextrin.

We did not specifically knockdown ER $\alpha$ 36 or ER $\beta$ 59, but our results strongly implicate both receptors in the failure of male cells to respond to E<sub>2</sub>. Male caveolae lack both isoforms but possess ER $\alpha$ 68 and ER $\alpha$ 46. Although agonists for both ER $\alpha$  and ER $\beta$  activated PKC in female chondrocytes, neither agonist was able to do so in male chondrocytes, despite the presence of two ER $\alpha$  isoforms as well as evidence components

of the signaling pathways involved in PKC activation were present and could be stimulated to the same extent as in female cells.

ER trafficking may be an important variable in the differential response of male and female chondrocytes to  $E_2$  as well. Others have shown that palmitoylation is required for the plasma membrane association of ERs [25, 113]. This was the case for female chondrocytes in the present study.  $E_2$  increased the association of ERs with the plasma membrane, particularly  $ER\alpha_{46}$ ,  $ER\alpha_{36}$  and  $ER\beta_{59}$  whereas tunicamycin reduced the association of all three  $ER\alpha$  isoforms and  $ER\beta_{59}$  with the plasma membrane. In male cells, however,  $E_2$  caused a marked reduction in the association of  $ER\alpha_{68}$ ,  $ER\alpha_{46}$  and  $ER\beta_{59}$  with the plasma membrane. Why the receptor trafficking in the male cells was contra to that of the female cells is not clear. In female cells,  $E_2$  elicits formation of a multi-receptor complex that involves both  $ER\alpha$  and  $ER\beta$ , but this is not the case for male cells, suggesting that a scaffolding unit may be absent. In other studies, caveolin-1 provides scaffolding for ER translocation to the plasma membrane [72, 218, 223] and palmitoylation is required for caveolin-1 to interact with the plasma membrane [224]. This suggests that a difference between male and female cells may be related to the palmitoylation mechanism. It is also possible that  $E_2$  treatment in male cells may lead to ER translocation to the nucleus.

Our results confirm our previous study that female cells have more ERs than male cells [129] and show that it is specifically the pool of ERs that are on the surface of the cell that contribute to this increase. The percentage of positive female cells was not significantly different than the percentage of positive male cells in non-permeabilized cells, although the total number of  $ER\alpha$  on the surface was significantly higher in female

cells. This is in agreement with our previous study, which showed that there was no difference in  $E_2$ -binding affinity between females and males, but females had more receptors [129]. No differences in total  $ER\alpha$  number were found when assessing  $ER\alpha$  content of permeabilized cells whereas  $ER\alpha$  content of non-permeabilized cells, i.e., the cell surface, was more than two times greater in female cells. This is an important observation given the fact that  $ER\alpha36$  was present only on the plasma membranes of female cells, whereas all other isoforms of  $ER\alpha$  as well as  $ER\beta59$  were present on the plasma membranes of male cells. While this strongly supports the hypothesis that  $ER\alpha36$  plays a critical role, it is not yet definitive proof. However, recent studies in our lab examining the role of  $ER\alpha36$  in  $E_2$  dependent activation of PKC in breast cancer cells showed that specific antibodies to this isoform blocked the stimulatory effect of the hormone [211].

$E_2$  activates PKC in growth plate chondrocytes occurs via two signaling pathways, PLC and  $PLA_2$ . Our results indicate that the failure of male cells to respond to  $E_2$  is not due to an intrinsic defect in either pathway. Activation of PLC *m*-3M3F $\beta$ S could be achieved to the same extent in male and female cells. Similarly, both male and female cells were capable of generating  $PGE_2$  when PLAA was used to activate  $PLA_2$ . The effect of PLAA was less robust in male cells, raising the possibility that the  $PLA_2$  pathway was reduced in some manner, but it is difficult to draw too many conclusions given the fact that  $PGE_2$  production is a downstream surrogate for the enzyme itself. These data do show that the difference in response occurs upstream from the enzymes triggering the signaling pathways and support the conclusion that the difference is at the receptor level.

This study demonstrates that DHT regulates growth plate chondrocytes in a sex-specific manner via rapid signaling pathways. The effects of DHT were observed only in chondrocytes from male rat growth plates and occurred within 9 minutes. Both PKC and PLA2 were activated in a dose-dependent manner and increases in activity were seen at physiologically relevant concentrations. No effect of the testosterone metabolite was observed in the female cells. Moreover, the results show that the stimulatory effects on these two signaling pathways were specific to DHT and not testosterone as testosterone had no effect on either enzyme activity.

Neither gene transcription nor translation were required for DHT-dependent PKC activation to occur, indicating that DHT stimulated existing enzyme. We did not determine which PKC isoform was sensitive to DHT, but the assay conditions that were used are optimized to isoforms sensitive to both  $\text{Ca}^{2+}$  ions and phospholipids, suggesting a role for PKC $\alpha$  in the mechanism [196, 197].

CPA, which competes with testosterone and DHT to bind to the androgen receptor, did not inhibit the rapid responses, suggesting that DHT regulates PKC via a specific receptor that is different than the classic nuclear receptor. T-BSA which can not enter the cell, also failed to activate PKC, indicating that testosterone does not participate via a membrane form of AR and suggesting that DHT's effect is also via membrane AR. It is likely that the DHT-receptor is membrane-associated, however, as DHT stimulated PKC by mechanisms that are membrane-mediated.

The DHT-receptor might be a G-protein coupled receptor, as our results showed that G-protein is required for DHT-dependent PKC activation. There are two classes of G-proteins that couple receptors to the activation of PI-PLC: one that is PTX-sensitive

and one that is PTX-insensitive [198]. Pre-incubation of chondrocytes with both PTX ( $G_{\alpha i}$  inhibitor) and CTX ( $G_{\alpha s}$  activator) completely blocked the stimulatory effect of DHT on PKC activity. In other systems, upon G-protein activation,  $G_{\alpha}$  disassociates from  $G\beta\gamma$ , which can then activate  $Ca^{2+}$  ion channels on the plasma membrane [199]. Our results show that DHT stimulates influx of extracellular  $Ca^{2+}$  ions. Moreover, inhibition of  $Ca^{2+}$  influx blocks the stimulatory effect of DHT on PKC.  $G_{\alpha}$  proteins have also been shown to activate PI-PLC [200],[201] and PLC was required for DHT-dependent PKC activation, since specific inhibition of PI-PLC blocked activation of PKC by DHT. Moreover, inhibition of release of  $Ca^{2+}$  ions from the endoplasmic reticulum also reduced DHT's effect. PI-PLC catalyzes the release of IP<sub>3</sub>, which opens these  $Ca^{2+}$  ion channels and resulting DAG translocates the active PKC to the plasma membrane. Although  $G_{\alpha q}$  is generally associated with PI-PLC activation [202],  $G_{\alpha i}$  has also been shown to do so [182].

It is also possible that  $G_{\alpha i}$  is activating PLA<sub>2</sub>, and that is upstream of PI-PLC, as we have shown for  $1\alpha,25$ -dihydroxy vitamin D<sub>3</sub>-dependent activation of PKC in growth plate chondrocytes [203]. Our results show that DHT activated PKC via a PLA<sub>2</sub>-dependent pathway. DHT caused a rapid increase in PLA<sub>2</sub> activity and inhibition of PLA<sub>2</sub> blocked the stimulatory effect of DHT on PKC. Production of lysophospholipid [204] and release of arachidonic acid [205] are catalyzed by PLA<sub>2</sub> via phospholipid hydrolysis. Arachidonic acid can act as co-factor for PKC $\alpha$  and also provides a substrate for constitutively active cyclooxygenase-1 (Cox-1) in growth plate chondrocytes, which leads to prostaglandin production. In addition, lysophospholipid can activate PLC [203], and can then initiate the downstream PLC-PKC pathway

Our results demonstrate that DHT regulates proliferation and differentiation of growth plate chondrocytes by multiple mechanisms. The fact that U73122 blocked the stimulatory effect of DHT on alkaline phosphatase activity suggests that rapid membrane response via the PLC-PKC pathway plays an important role in differentiation of male chondrocytes. In contrast, DNA synthesis is not regulated in this manner. We previously showed that testosterone and DHT can regulate proliferation [33]; however, in the present study, a 9 minute pulse of DHT had no effect on [<sup>3</sup>H]-thymidine incorporation, indicating that the proliferative effects of testosterone and/or DHT are likely mediated by the classic androgen receptor.

Rat growth plate chondrocytes possess 5 $\alpha$ -reductase and convert testosterone to DHT [33, 172], and regulation of these cells by testosterone is dependent on the production and release of DHT [172]. Here we show that DHT but not testosterone elicits the rapid membrane response. Taken together, this suggests that the membrane-associated effects of androgens are mediated by paracrine actions of DHT.

In summary, DHT regulates male resting zone chondrocytes through rapid activation of a membrane-associated receptor. This results in PLA2 and PLC-mediated activation of PKC and increase in chondrocyte differentiation. These findings indicate the importance of the membrane effects as mediators of DHT action in chondrocytes and suggest the existence of a specific membrane receptor for DHT in chondrocytes.

This work has established the membrane receptors involved in 17 $\beta$ -estradiol (E2) and dihydrotestosterone sexual-dimorphic effect on longitudinal bone growth.

Additionally we were able to identify and understand the non-traditional steroid signaling mechanism. Moreover, it may lead to design compounds that only activate the membrane steroid receptors and not the traditional cytoplasmic receptors. This could be very beneficial for the treatment of postmenopausal osteoarthritis and osteoporosis while not increasing the risks commonly associated with hormone replacement therapy like increased incidence of breast and ovarian cancers. However, there are still numerous unanswered questions regarding sexual dimorphism and cartilage regeneration.

The most important next step is applying our finding on *in vivo* studies to determine what role E2 and DHT could have on *in vivo* tissue formation. It would also be of interest to investigate if this sex-specific E2 membrane effect was present in all tissues including other types of cartilage like elastic auricular tissue or intervertebral discs. Perhaps different tissues express variable concentrations and isoforms of sex steroid membrane receptors that control their sexual dimorphism. If this is the case, then the development of drugs that are more beneficial for only men or women may be a reality in the future.

Our previous studies demonstrated that DHT can rapidly activate PKC via a pathway that involves G-protein-dependent PI-PLC only in male chondrocytes. However, the actual receptors that mediate DHT-stimulated PKC were not determined. That the effect of DHT is receptor mediated was demonstrated in the present study by the fact that only DHT but not *ent*-DHT increased PKC activity. Because enantiomers are structural mirror images with identical physicochemical properties and receptors have well-defined and structurally discriminate binding pockets, they can generally identify specific ligands of different absolute configurations. In contrast, membrane lipids present a dynamic environment that does not maintain structurally well-defined binding sites for steroids

[102]. Thus, the difference in the effect of each enantiomer (DHT and *ent*-DHT) on the cell response implies that the binding affinity of DHT to the specific receptor is more effective than its enantiomer (*ent*-DHT).

Our results demonstrate that both ER $\alpha$  and ER $\beta$  are involved in mediating the rapid membrane-associated effects of DHT on the PKC signaling pathway. Antibodies specific for ER $\alpha$  and ER $\beta$  blocked the stimulatory effects of DHT on PKC activity. ER $\alpha$  [105] and ER $\beta$  [106], as well as truncated forms of both receptors are present in the plasma membrane [107, 108] and caveolae [109, 110], implying that membrane forms of these receptors may play a role in mediating rapid responses to the hormone via caveolae-dependent mechanisms. This is supported by the observation that destruction of caveolae with  $\beta$ -CD blocked the stimulatory effect of DHT on PKC.

Further evidence for the role of caveolae in mediating the effects of DHT on PKC, is translocation of the AR to this specialized membrane domain in response to the hormones. ARs do not display an intrinsic transmembrane domain [219]; thus an interaction of ARs with specific membrane proteins has been proposed to explain their membrane localization [219, 220]. The AR has been shown to interact with caveolin-1 in other systems [221]. We did not address this specifically but our finding supports this hypothesis. Caveolin-1 exists primarily in fraction 3 of the plasma membrane and AR was present in fraction 3 only after treatment with DHT.

We found that posttranslational palmitoylation is required for AR membrane localization and for DHT-induced recruitment of ARs to caveolae. It is possible that by palmitoylation, ARs are dynamically recruited from the cytoplasmic pool to the plasma membrane and specifically to caveolae, which contain other signaling molecules



including G-protein  $\alpha$ -subunits, kinases, and caveolins [114]. Palmitoylation seems to be a common regulatory mechanism in the formation of membrane-associated signaling complexes. The interaction between palmitoylated ARs and other caveolae proteins may act as a signal for the directional recruitment and restructuring of the receptor. .

This study demonstrates that DHT signaling via the AR plays a role in chondrocyte maturation by increasing [ $^{35}\text{S}$ ]-sulfate incorporation, suggesting an increase in the synthesis of a sulfated proteoglycan extracellular matrix. Alkaline phosphatase specific activity also increased [23], supporting the hypothesis that the chondrocytes produce a mature matrix containing alkaline phosphatase-rich extracellular matrix vesicles [222] in response to DHT. Importantly, our data demonstrate that this effect of DHT is mediated by AR-dependent signaling. It was blocked by AR antagonist, Casodex and by the PLC inhibitor U73122.

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